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(54) Title: TUMOR NECROSIS FACTOR ALPHA CONVERTASE

(57) Abstract

The present invention relates to tumor necrosis factor alpha (TNFα), and more specifically to the enzyme TNFα-convertase (TNFα-con) that can proteolytically convert TNFα precursor to mature TNFα. The present invention provides DNA sequences encoding mammalian TNFα-con and functional equivalents thereof, recombinant expression vectors comprising said DNA sequences, host cell lines comprising said expression vectors, inhibitors of TNFα-con, inhibitors modified for use as ligands for affinity purification of TNFα-con, and methods for treating diseases or conditions resulting from abnormal levels of TNFα in a mammalian subject.

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TUMOR NECROSIS FACTOR ALPHA CONVERTASE

1. FIELD OF THE INVENTION

The present invention relates to tumor necrosis factor alpha (TNFα), and more specifically to the enzyme TNFα convertase (TNFα-con) that can proteolytically convert TNFα precursor to mature TNFα. The present invention provides DNA sequences encoding mammalian TNFα-con and functional equivalents thereof, recombinant expression vectors and host cells comprising said DNA sequences, methods for making TNFα-con using such DNA sequences, inhibitors of TNFα-con, inhibitors modified for use as ligands for affinity purification of TNFα-con, and methods for treating diseases or conditions resulting from abnormal levels of TNFα in a mammalian subject.

The invention is demonstrated by a working example in which TNFα-con is isolated and a cDNA encoding human TNFα-con is cloned and sequenced.

2. BACKGROUND OF THE INVENTION

TNFα, also known as cachectin, is a mammalian protein that is produced primarily by activated monocytes and macrophages. TNFα is a potent cytokine that plays a pivotal role in host defense against invasion by microorganisms by mediating cellular responses to infection. TNFα is generally not present in measurable amounts in normal mammalian sera, but appears rapidly in response to several types of stimuli, including infection by viruses or bacteria, trypanosoma and plasmodia, and the cytokine IL-1 (Beutler and Cerami, 1989, Ann. Rev. Immunol. 7:625-655). The most potent known stimulus of TNFα production is bacterial lipopolysaccharide (LPS).

TNFα is an important endogenous factor in the pathogenesis of septic shock (Williams and Summers, 1994, Exp. Opin. Invest. Drugs 3:1051-1056), and in chronic wasting (cachexia) associated with acute inflammatory or malignant diseases (Vassali, 1992, Ann. Rev. Immunol. 10:411-452;

Beutler and Cerami, 1989, above). TNFα has been recognized as manifesting a dose dependent toxicity. For example, if TNFα is present at high levels even for a short period of time, it may trigger septic shock. If TNFα is present at low levels for too long a period of time, it may result in cachexia.

Abnormal levels of TNFα have also been implicated in the pathogenesis of the following diseases or conditions: systemic inflammatory response syndrome, reperfusion injury, cardiovascular disease, infectious disease, obstetrical or gynecological disorders, inflammatory disease or autoimmunity, allergic or atopic diseases, malignancies, transplant complications, and others. More specifically, abnormal levels of TNFα appear to play a pathogenic role in AIDS (Folks *et al.*, 1989, Proc. Natl. Acad. Sci. *USA* 86:2365); graft-versus-host disease (Piguet *et al.*, 1987, J. Exp. Med. 166:1280); cerebral malaria (Grau *et al.*, 1987, Science 237:1210); and rheumatoid arthritis (Brennan *et al.*, 1989, Lancet ii:244; Elliot *et al.*, 1994, Lancet 344:1105), among others.

Human TNFα is initially synthesized as a membrane-bound precursor of approximately 26 kDa. A soluble mature 17 kDa peptide is released from the precursor after enzymatic cleavage by TNFα-con of the bond between TNFα precursor residues Ala_{76} and Val_{77}. The TNFα precursor lacks a standard signal sequence. However, secretory vesicle transport events may be coupled to processing since mutation of the Ala_{76}-Val_{77} cleavage site prevents secretion (Perez *et al.*, 1990, Cell 63:251-258).

Mohler *et al.*, 1994, Nature 370:218-220, partially purified TNFα-con from a human monocytic cell line, THP-1, and showed it to be a Zn^{2+}-dependent metalloproteinase. Gearing *et al.*, 1994, Nature 370:555-557, demonstrated that matrix metalloproteinases, such as collagenase, could also process TNFα precursor at the correct cleavage sequence. In addition, inhibitors of proteases, such as serine proteases, serine/cysteine proteases, cysteine proteases and aspartyl

proteases, were shown to be ineffective at inhibiting TNFα-con activity. McGeehan *et al.*, 1994, Nature, 370:558-561, showed that compound GI 129471, a hydroxamic acid-related inhibitor, which targets the highly conserved Zn^{2+} binding motif, HEXGH, of metalloproteinases, is a specific inhibitor of TNFα-con activity. Finally, Becherer *et al.*, 1995, J. Cell. Biochem. Supp. 0(19B):253, reported TNFα-con enzymatic activity in a microsomal preparation that was inhibited by GI 129471, and the isolation of a putative TNFα-con by photoaffinity chromatography utilizing a photoreactive crosslinking derivative of GI 129471.

Various approaches have been proposed to control TNFα levels in patient sera, including the use of monoclonal or chimeric antibodies to bind and neutralize excess TNFα. See, for example, U.S. Pat. No. 5,231,024; U.S. Pat. No. 5,360,716; U.S. Pat. No. 5,436,154; U.S. Pat. No. 5,447,851; WO 92/11383; EP No. 0 366 043; EP No. 0 492 448 A1; and EP No. 0 585 705 A1. An alternative approach to bind up excess TNFα is to administer modified versions of the TNFα receptor. See WO 92/07076; WO 94/06476; and EP No. 0 418 014 A1. One difficulty with these approaches involves the potential for eliciting an immune reaction against the administered antibody or peptide molecule. Alternatively, U.S. Pat. No. 5,344,915 discloses the isolation of soluble TNFα binding proteins from human urine.

A further approach is the expression of TNFα anti-sense mRNA in cells that otherwise produce TNFα to bind to and prevent the translation of TNFα precursor mRNA. See No. EP 0 414 607 A2.

Yet another approach involves identifying and administering chemical compounds that specifically inhibit the synthesis or cellular secretion of TNFα. U.S. Patent No. 5,385,901 discloses the use of thalidomide and related compounds to specifically inhibit the production of TNFα. Mohler *et al.*, 1994, above, specifically inhibited the release of TNFα using hydroxamic acid-related compounds, and were thereby able to increase the rate of survival of mice

injected with an otherwise lethal dose of LPS plus D-galactosamine. Gearing *et al.*, 1994, above, and McGeehan *et al.*, 1994, above, both identified additional hydroxamic acid-related inhibitors that specifically blocked TNFα secretion. See also WO 95/06031.

Screening for compounds that inhibit TNFα-con would be facilitated by a readily available and abundant source of purified TNFα-con. Such inhibitors would be useful to reduce or otherwise modulate TNFα levels in a mammalian subject, thereby treating diseases or conditions resulting from abnormal levels of TNFα. In addition, the availability of large quantities of TNFα-con would facilitate the development and identification of derivatives, analogs and peptides of TNFα-con that could serve to modulate TNFα levels in a mammalian subject in need of such treatment. Accordingly, it would be useful to provide compositions and methods with which to produce large quantities of isolated TNFα-con.

3. SUMMARY OF THE INVENTION

The present invention is directed to TNFα, and more specifically to TNFα-con having biological activity to convert TNFα precursor to mature TNFα. The present invention provides cDNA sequences encoding enzymatically active mammalian TNFα-con, and more specifically a cDNA sequence encoding human TNFα-con. The present invention also provides DNA sequences encoding derivatives, analogs or peptides of mammalian TNFα-con polypeptides that are substantially similar to mammalian TNFα-con and that exhibit biological activity.

The present invention further provides recombinant expression vectors comprising said DNA sequences, host cell lines comprising said DNA sequences or expression vectors, and recombinantly expressed, enzymatically active TNFα-con, or functional equivalents thereof.

The present invention further provides compounds that inhibit the biological activity of TNFα-con, which may be useful for treating diseases or conditions related to abnormal levels of TNFα.

The present invention further provides novel modified inhibitors for use as ligands in the affinity purification of TNFα-con.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. cDNA sequence (SEQ ID NO 1) encoding human TNFα-con and corresponding deduced amino acid sequence (SEQ ID NO 2). Asterisks show the termination codon. Amino acid residues 405-409 represent the conserved sequence of the zinc-binding motif of metalloproteinases.

FIG. 2A. Enzyme activity, as determined by cleavage of a synthetic substrate, of fractions collected from a glycerol gradient after affinity purification of porcine TNFα-con. FIG. 2B. SDS-PAGE analysis of fractions collected from the glycerol gradient corresponding to those tested in FIG. 2A.

FIG. 3. Reducing SDS-PAGE analysis of porcine TNFα-con before and after deglycosylation.

FIG. 4A. Enzyme activity, as determined by cleavage of a synthetic substrate, of fractions collected from a glycerol gradient after affinity purification of human TNFα-con. FIG. 4B. SDS-PAGE analysis of fractions collected from the glycerol gradient corresponding to those tested in FIG. 4A.

FIG. 5. A contiguous mapping of clones psC-1, psC-2, psC-3 and psC-5, obtained by screening a porcine spleen cDNA library with probes specific to the porcine TNFα-con coding sequence. Sequence comparison showed that the four clones overlap. The map represents the entire length of 2,414 bases from the four clones.

FIG. 6. cDNA sequence (SEQ ID NO 9) encoding a portion of the major open reading frame of porcine TNFα-con and corresponding deduced partial amino acid sequence (SEQ ID

NO 10). Asterisks show the termination codon. Amino acids 8-12 represent the conserved sequence of the zinc-binding motif of metalloproteinases.

FIG. 7. Contiguous map of positive clones isolated from cDNA libraries from human leukocytes (hc7, hc9, hc11), and from human monocytes (3'#1, 3'#4, 3'#5, 5'#4, 5'#7). hc7 contains two internal deletions at base pairs 525-613 and 2156-2294.

FIG. 8. Structure of a biotinylated hydroxamic acid-related compound useful in the affinity purification of TNFα-con.

FIG. 9. Stepwise (A-I) synthesis of a biotinylated hydroxamic acid-related compound (I).

FIG. 10. Stepwise synthesis of reagent C used in the synthesis procedure shown in FIG. 9.

FIG. 11. Sequence of RACE14 clone (SEQ ID NO 39).

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. DNA SEQUENCES ENCODING TNFα-CON

The nucleic acid sequences which can be used in accordance with the invention include but are not limited to any nucleic acid sequence that encodes a TNFα-con or a functional equivalent thereof, including: (a) any nucleotide sequence that is complementary to a nucleotide sequence that hybridizes to a mammalian TNFα-con coding sequence under highly stringent conditions, *e.g.*, washing in 0.1 x SSC/0.1 % SDS at 68°C (Ausubel *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Greene Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at page 2.10.3.), and encodes a product that exhibits a biological activity characteristic of a mammalian TNFα-con; (b) any nucleotide sequence that hybridizes to a nucleotide sequence that is complementary to a mammalian TNFα-con coding sequence under less stringent conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2 x

SSC/0.1 % SDS at 42°C (Ausubel *et al.*, 1989, above), and encodes a product that exhibits a biological activity characteristic of a mammalian TNFα-con; and (c) any nucleotide sequence that encodes a product that exhibits a biological activity characteristic of a mammalian TNFα-con. As used herein, the term "TNFα-con" refers to a naturally occurring mammalian TNFα-con polypeptide and all functional equivalents thereof, unless otherwise noted.

For purposes of the present invention, "functional equivalents" of TNFα-con encompass all derivatives, analogs and peptides, as those terms are used in the art, of a mammalian TNFα-con that are substantially similar to the mammalian TNFα-con polypeptide and exhibit a biological activity of a mammalian TNFα-con.

As used herein, the term "substantially similar" means that a particular amino acid sequence varies from the amino acid sequence of a naturally occurring mammalian TNFα-con sequence by one or more amino acid substitutions, deletions, additions, or truncations, or by the addition of one or more chemical functional groups, or by some combination thereof. A peptide or polypeptide is considered to be substantially similar to a mammalian TNFα-con if its amino acid sequence is at least about 50% homologous to the corresponding amino acid sequence of the mammalian TNFα-con, and more preferably is at least about 80% homologous to the corresponding amino acid sequence of the mammalian TNFα-con. A polypeptide that is substantially similar to a mammalian TNFα-con will also preferably exhibit at least one type of biological activity characteristic of a mammalian TNFα-con.

For the purposes of this invention, "biological activity" as applied to TNFα-con encompasses: (1) the ability to proteolytically cleave a mammalian TNFα precursor at a cleavage site corresponding to the peptide

bond between residues Ala_{76} and Val_{77} of human TNFα precursor; or (2) the ability to cleave an equivalent peptide bond in a synthetic substrate; or (3) the ability to detectably bind to TNFα precursor polypeptide, to TNFα, or to a synthetic substrate comprising an equivalent peptide bond which is cleavable by a mammalian TNFα-con.

Any mammalian tissue or cell can serve as a source of a nucleotide sequence encoding TNFα-con for use in molecular cloning. Since TNFα-con activity is required for the processing of TNFα precursor to mature TNFα, it can reasonably be inferred that any tissues or cells that produce mature TNFα will serve as a source for TNFα-con mRNA and TNFα-con polypeptide. For example, it is known that TNFα is produced in a wide range of mammalian tissues and cells in response to various types of stimulation, including exposure to LPS. Such mammalian tissues include but are not limited to spleen and thymus. Specific mammalian cell types include but are not limited to macrophages, monocytes, T-lymphocytes, β-lymphocytes, mast cells, polymorphonuclear leukocytes, keratinocytes, astrocytes, microglial cells, smooth muscle cells, intestinal paneth cells, and tumor cells including fibrosarcomas, epithelial tumor lines, myelomas, T-cell leukemias, and the myeloid progenitors of acute and chronic myeloid leukemias, among others (Vassalli, 1992, above; Beutler and Cerami, 1989, above). For example, TNFα-con mRNA and polypeptides can be purified from LPS-stimulated cells of murine macrophage cell line RAW 264.7, which can be obtained from the American Type Culture Collection (Rockville, Md.) (Accession No. TIB 71) (Jue *et al.*, 1990, Biochemistry 29:8371-8377).

Likewise, any human cell can serve as a source of a nucleotide sequence encoding TNFα-con for molecular cloning. For example, TNFα-con mRNA and polypeptide can be obtained from human monocytes which can be purified

from human blood, as for example by density centrifugation (Kriegler *et al.*, 1988, Cell 53:45-53). An established human monocytic cell line that can be used as a source of TNFα-con mRNA and polypeptide is THP-1, which can be obtained from the ATCC (Accession No. TIB 202).

The production of TNFα in any of the above cell lines or tissues can be determined either immunologically using anti-TNFα antibodies, which may be produced by standard methods, or by utilizing any of several bioassays known in the art including, for example, an *in vitro* cytotoxicity assay using L-929 murine fibroblast cells, as described by Matthews and Neale, 1987, in: Clemens *et al.* (eds), Lymphokines and Interferons: A Practical Approach, IRL, Oxford, pp. 221-225, which is incorporated herein by reference.

A nucleotide sequence encoding TNFα-con may be isolated from any of the above described tissues or cells by known methods, including but not limited to reverse transcriptase-polymerase chain reaction (RT-PCR) from total mRNA to produce cDNA, or by screening cloned genomic DNA (*e.g.*, a DNA library) with probes unique to the TNFα-con gene sequence. For example, a labelled probe derived from a TNFα-con cDNA may be used to isolate a TNFα-con related gene by screening a genomic library. For example, using techniques known in the art, total mRNA can be isolated from any of the aforementioned tissues or cell types and reverse transcribed to produce cDNA, which is then screened, for example, with a labelled probe. Such a probe can be designed based, for example, on a partial amino acid sequence of a TNFα-con polypeptide from the same or a different mammalian species, taking into account the genetic code and its known degeneracy.

TNFα-con can be isolated from any of the above-listed tissues or cells such as, for example, from mammalian spleen tissue according to procedures described

in Section 6.1 below. Briefly, mammalian spleen tissue is ground up in an appropriate buffer containing protease inhibitors at 4°C to obtain, through a series of steps, a membrane preparation which is then passed through a concanavalin A (conA) column, which binds TNFα-con. Further purification of the eluted enzyme typically requires one or more affinity chromatography steps such as, for example, by contacting a partially purified TNFα-con preparation with a modified inhibitor of TNFα-con under conditions that allow the binding of TNFα-con to the inhibitor, and isolating the TNFα-con-inhibitor conjugate.

A novel modified inhibitor is an hydroxamic acid-related inhibitor having the formula shown in FIG. 8, where R comprises a moiety that can be used for binding to a further compound so as to isolate any TNFα-con that binds to the inhibitor. For example, the moiety may be biotin. R may further comprise a spacer arm between the inhibitor and the moiety. The spacer arm can be any chain length and may incorporate a disulfide bond between the hydroxamic acid inhibitor and the biotin moiety. For example, but not by way of limitation, R can be any of the following: $-(CH_2)_n$-Biotin, where n = 0-10; $-(CH_2)_n$-Imino Biotin, where n = 0-10; $-(CH_2)_n$-S-S-$(CH_2)_n$-Biotin, where n = 0-10; or $-(CH_2)_n$-S-S-(CH_2)n-Imino Biotin, where n = 0-10. Biotinylation of the inhibitor can be carried out by known methods. Preparation of the specific biotinylated inhibitor shown in FIG. 8 is described below (Section 7). Additionally, the present invention encompasses the use of derivatives of biotin that may improve solubility or increase affinity for streptavidin.

Alternatively, TNFα-con can be isolated by photoaffinity chromatography using known methods and utilizing, for example, a photoreactive crosslinking derivative of an inhibitor of TNFα-con, such as GI 129471, bound to a solid phase matrix.

TNFα-con isolated from porcine spleen has an apparent mass of about 85 kDa, which drops to about 62 kDa after deglycosylation.

Once the polypeptide is isolated, a complete or partial amino acid sequence of the polypeptide may be obtained, *e.g.*, by the Edman degradation procedure (see Creighton, 1983, Proteins, Structures And Molecular Principles, W.H. Freeman & Co., N.Y., pp. 34-49). Degenerate oligonucleotide primers may then be designed based on the complete or partial amino acid sequence, and used in a PCR to amplify a portion of the TNFα-con coding sequence from either a genomic or cDNA library. The amplified portion may then be used as a probe to obtain the full nucleotide coding sequence for TNFα-con.

For example, the following degenerate oligonucleotide PCR primers, designed as based on a 41 amino acid fragment of porcine TNFα-con, are useful to amplify a portion of a mammalian TNFα-con coding sequence: primer conv-1, having sequence 5′-GTI CA(A/G) GA(T/C) GT(A/G) AT(T/C/A) GA-3′(SEQ ID NO 3); primer conv-2, having sequence 5′-GTI CA(A/G) GA(T/C) GT(T/C) AT(T/C/A) GA-3′(SEQ ID NO 4); and primer conv-3, having sequence 5′-CC IAC (A/G/T)AT (A/G)TT (A/G)TC (T/C)GC-3′ (SEQ ID NO 5). For example, either primer conv-1 (SEQ ID NO 3) or primer conv-2 (SEQ ID NO 4) can be used in combination with primer conv-3 (SEQ ID NO 5) to amplify by RT-PCR an 89 bp fragment (SEQ ID NO 8) from porcine spleen poly(A+) RNA encoding TNFα-con.

PCR amplification may be carried out by known methods. See, for example, the techniques described in Innis *et al.* (eds), 1995, PCR Strategies, Academic Press, Inc., San Diego; and Erlich (ed) 1992, PCR Technology, Oxford University Press, New York, which are incorporated herein by reference. A PCR mixture comprising any suitable primers, the nucleotide sequence to be amplified, and appropriate enzymes and buffers, are processed according to standard PCR protocols to amplify

the DNA sequence. Amplification may be carried out, for example, on a cDNA library that has been prepared by reverse transcription of porcine spleen poly(A+) mRNA using commercially available reagents such as, for example, a cDNA cycle kit from Invitrogen. The sequence of any amplified product may be determined to confirm that it corresponds to the complete or partial amino acid sequence of a TNFα-con. Such amplified sequences may then be used to screen for TNFα-con coding sequences in any mammalian cDNA or genomic library, including human. Once obtained, the full TNFα-con sequence can be characterized and used in molecular cloning, as described below.

In the molecular cloning of a TNFα-con DNA sequence, DNA fragments are typically generated, some of which will encode the desired TNFα-con sequence. To produce these fragments, the DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may fragment DNA in the presence of manganese, or physically shear the DNA, as for example by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to agarose and polyacrylamide gel electrophoresis (PAGE) and column chromatography.

Once DNA fragments are generated, identification of one or more specific DNA fragments containing the TNFα-con DNA sequence may be accomplished in a number of ways. For example, if an amount of a TNFα-con gene or its specific RNA, or a portion thereof, is detectably labeled, the generated DNA fragments may be screened by hybridization to the labeled probe.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the invention for the cloning and expression of a TNFα-con protein. Such DNA sequences include those capable of hybridizing to a nucleotide

sequence that is complementary to the TNFα-con coding sequence under highly or moderately stringent conditions, as defined above. Stringency conditions may be adjusted in a number of ways. For example, when performing PCR, the temperature at which annealing of primers to template takes place and/or the concentration of $MgCl_2$ in the reaction buffer may be adjusted. When using radioactively labeled DNA fragments or oligonucleotides in hybridization reactions, stringency may be adjusted by changes in the ionic strength of the wash solutions and/or by careful control of the temperature at which the washes are carried out.

Altered nucleotide sequences which may be used in accordance with the invention include those comprising deletions, additions or substitutions of different nucleotides resulting in a sequence that encodes the same or a functionally equivalent gene product. Alterations in the nucleotide sequence may result in changes, *i.e.*, deletions, additions, substitutions or truncations, in the amino acid sequence that may or may not be silent, but which produce a product that exhibits a biological activity characteristic of TNFα-con. Such nucleotide changes may be made taking into account similarities in the polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the amino acid residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine. Amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

Once a DNA sequence encoding TNFα-con is isolated, it can be amplified by any methods known in the art, including by chemical synthesis, PCR amplification, or cloning in a host cell. See, for example, the

techniques described in Maniatis *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.; and Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which are incorporated herein by reference.

Utilizing PCR procedures and other techniques, a cDNA encoding human TNFα-con has now been cloned and sequenced (SEQ ID NO 1) (FIG. 1) that encodes the amino acid sequence (SEQ ID NO 2) also shown therein. In a specific embodiment of the invention (see Section 6, below), the cDNA sequence for human TNFα-con was obtained by first purifying porcine TNFα-con, determining a partial amino acid sequence thereof, synthesizing degenerate PCR primers based on the partial porcine amino acid sequence, amplifying a fragment of the porcine TNFα-con DNA coding region, and using the fragment as a probe to screen human leukocyte and monocyte cDNA libraries so as to isolate a full length cDNA sequence encoding human TNFα-con.

Once isolated, the TNFα-con DNA sequence of the invention can be analyzed by known methods, including but not limited to Southern hybridization, Northern hybridization, restriction endonuclease mapping, and DNA sequence analysis. Southern hybridization with a TNFα-con specific probe can allow the detection of the TNFα-con gene, either natural or introduced, in various cell types. Northern hybridization analysis can be used to determine the expression of the TNFα-con gene in different cell types, or at various stages of development or induction, for example. The stringency of the hybridization conditions for both Southern and Northern hybridization can be manipulated to ensure detection of

nucleic acids with the desired degree of relatedness to the specific TNFα-con probe used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of the TNFα-con gene, and the extent of homology between the TNFα-con gene and other genes. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger *et al.*, 1977, Proc. Natl. Acad. Sci. *USA* 74:5463), or use of an automated DNA sequenator (*e.g.*, Applied Biosystems, Foster City, Ca.).

5.2. VECTORS TO DIRECT EXPRESSION OF DNA SEQUENCES ENCODING TNFα-CON

Once a DNA sequence encoding TNFα-con is obtained, it may be transferred directly into a host cell, or first inserted into an appropriate expression vector which is then transferred to a host cell, for propagation and expression. Such a vector is preferably constructed so that the TNFα-con coding sequence is in operative association with one or more regulatory elements necessary for transcription and translation of the coding sequence and production of a biologically active molecule.

As used herein, the term "regulatory element" includes but is not limited to inducible and non-inducible promoters, enhancers, operators and other elements known in the art that serve to drive and/or regulate expression. Also, as used herein, a DNA coding sequence is in "operative association" with one or more regulatory elements where the regulatory elements effectively regulate and allow for the transcription of

the DNA coding sequence and/or the translation of its mRNA.

Methods known in the art can be used to construct expression vectors containing the TNFα-con DNA sequence in operative association with appropriate regulatory elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Maniatis, *et al.*, 1989, above; Ausubel *et al.*, 1989, above; and Sambrook *et al.*, 1989, above.

A variety of host expression vector systems, preferably those which contain the necessary regulatory elements for directing the replication, transcription, and translation of a TNFα-con coding sequence, may be utilized equally well by those skilled in the art to express the TNFα-con coding sequence. These host expression vector systems include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the TNFα-con coding sequence; yeast transformed with recombinant yeast expression vectors containing the TNFα-con coding sequence; insect cell systems infected with recombinant virus expression vectors, *e.g.*, baculovirus, containing the TNFα-con coding sequence; or animal cell systems infected with recombinant virus expression vectors, *e.g.*, adenovirus or vaccinia virus containing the TNFα-con sequence, and include cell lines engineered to contain, for example, multiple copies of the TNFα-con coding sequence either stably amplified, *e.g.*, CHO/dhfr, or unstably amplified in double-minute chromosomes, *e.g.*, murine cell lines.

The regulatory elements of these vectors may vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements may be

used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, *e.g.*, mouse metallothionein promoter, or from viruses that grow in these cells, *e.g.*, vaccinia virus 7.5K promoter or Moloney murine sarcoma virus long terminal repeat, may be used. Promoters obtained by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Illustrative transcriptional regulatory regions or promoters include for bacteria, the β-gal promoter, the T7 promoter, the TAC promoter, λ left and right promoters, trp and lac promoters, trp-lac fusion promoters, *etc.*; for yeast, glycolytic enzyme promoters, such as ADH-I and -II promoters, GPK promoter, PGI promoter, TRP promoter, *etc.*; for mammalian cells, SV40 early and late promoters, adenovirus major late promoters, *etc.*

Specific initiation signals are also required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire TNFα-con gene, including its own initiation codon and adjacent sequences, are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the TNFα-con coding sequences to ensure in-frame translation of the entire insert. These exogenous translational control signals and initiation codons can be obtained from a variety of sources, both natural and synthetic. In addition, the efficiency of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, *etc.* For example, in cases where an adenovirus is used as an expression vector, the

TNFα-con coding sequence may be ligated to an adenovirus transcription/ translation control complex, *e.g.*, the late promoter and tripartite ladder sequence. This chimeric gene may then be inserted into the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome, *e.g.*, region E3 or E4, will result in a recombinant virus that is viable and capable of expressing TNFα-con in infected hosts. Similarly, the vaccinia 7.5K promoter may be used.

An alternative expression system which could be used to express TNFα-con is an insect system such as, for example, where *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express the foreign sequence. The virus grows in *Spodoptera frugiperda* cells. The TNFα-con coding sequence may be cloned into a non-essential region, *e.g.*, the polyhedrin gene of the virus, and placed under the control of an AcNPV promoter such as, for example, the polyhedrin promoter. Successful insertion of the TNFα-con coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus, *i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene. These recombinant viruses may then be used to infect *Spodoptera frugiperda* cells in which the inserted gene is to be expressed.

Alternatively, retroviral vectors prepared in amphotropic packaging cell lines permit high efficiency expression in numerous cells types. This method allows the assessment of cell-type specific processing, regulation, or function of the inserted protein coding sequence.

A host cell strain may be chosen which modulates the expression of the inserted sequence, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, *e.g.*,

zinc and cadmium ions for metallothionein promoters. Expression of the genetically engineered TNFα-con may thus be controlled. This is important if the protein product of the cloned foreign gene is lethal to host cells. Furthermore, modifications such as, for example, phosphorylation or glycosylation, and processing, such as cleavage of protein products may be important for the biological activity of the protein. Different host cells have characteristic and specific mechanisms for the post-translational modification and processing of an expressed polypeptide. For example, modifications in the glycosylation pattern may be important for different functions of the protein. Thus, expression in a bacterial system will produce an unglycosylated "core" protein product. Expression in yeast will produce a glycosylated product. Expression in a mammalian cell, *e.g.*, in COS cells, can be used to ensure "native" glycosylation of the heterologous TNFα-con polypeptide. Such variations may or may not result in changes to one or more biological properties of the polypeptide. For example, it is possible that a TNFα-con polypeptide expressed in a particular cell type will retain the ability to bind to, but lack the ability to cleave, TNFα precursor. Such variously processed TNFα-con polypeptides fall within the scope of the present invention. Thus, the cell line or host system may be chosen to ensure the desired modification and processing of the expressed protein.

Fusion protein expression vectors may be used to express a TNFα-con fusion protein. The purified TNFα-con fusion protein may be used to raise antisera against the TNFα-con protein, to study the biochemical properties of the TNFα-con protein, to engineer TNFα-con fusion proteins with different enzymatic activities, or to aid in the identification or purification of the expressed protein. Possible fusion protein expression vectors include but are not limited to vectors that express β-

galactosidase and trpE fusions, maltose-binding protein fusions, glutathione-S-transferase fusions and polyhistidine fusions (carrier regions). Methods known in the art can be used to construct expression vectors coding for such TNFα-con fusion proteins. See, for example, the techniques described in Maniatis, *et al.*, 1989, above; Ausubel *et al.*, 1989, above; and Sambrook *et al.*, 1989, above.

The TNFα-con fusion protein may comprise a region that may be used for purification. For example, amylose resin may be used for purification of maltose binding protein fusions, or glutathione-agarose beads may be used for purification of glutathione-S-transferase fusion proteins, or divalent nickel resin can be used for the purification of polyhistidine fusions. Alternatively, antibodies against a carrier protein or peptide may be used for affinity chromatography purification of the fusion protein. For example, a nucleotide sequence coding for the target epitope of a monoclonal antibody may be engineered into the expression vector in operative association with the regulatory elements and situated so that the expressed epitope is fused to the TNFα-con polypeptide. For example, but not by way of limitation, a nucleotide sequence coding for the FLAG™ epitope tag (International Biotechnologies Inc., IBI), which is a hydrophilic marker peptide, can be inserted by standard techniques into the expression vector at a point corresponding, for example, to the carboxyl terminus of the TNFα-con polypeptide. The expressed TNFα-con-FLAG™ epitope fusion product may then be detected and affinity-purified using commercially available anti-FLAG™ antibodies (IBI).

The expression vector may also be engineered to contain polylinker sequences that encode specific protease cleavage sites so that any cloned protein may be released from the carrier region by treatment with a specific protease. For example, DNA sequences encoding

the thrombin or factor Xa cleavage sites may be included in the fusion protein vectors.

A signal sequence upstream from and in reading frame with the polypeptide coding sequence may be engineered into the expression vector by known methods to direct the trafficking and secretion of the expressed protein. Non-limiting examples of signal sequences include those from α-factor, immunoglobulins, outer membrane proteins, penicillinase and T-cell receptors, among others.

To aid in the selection of transformed or transfected host cells, the expression vector may be engineered to further comprise a coding sequence for a reporter gene product or other selectable marker. Such a coding sequence should preferably be in operative association with the regulatory element coding sequences as described above. Reporter genes which may be useful in the invention are well-known in the art and include those encoding chloramphenicol acetyltransferase (CAT), firefly luciferase, and human growth hormone, among others. Coding sequences that encode selectable markers useful in the invention are also well-known in the art and include those that encode gene products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement. Examples of such sequences include those that encode thymidine kinase activity or resistance to methotrexate, among others.

The expression vector can be additionally engineered according to known methods to enhance or optimize polypeptide expression, such as by mutating DNA regulatory elements to increase promoter strength or to alter the polypeptide coding sequence itself. Other modifications may include deleting intron sequences or excess non-coding sequences from the 5′ and/or 3′ ends of the polypeptide coding sequence in order to minimize sequence- or distance-associated negative effects on

expression of the polypeptide, *e.g.*, by minimizing or eliminating message destabilizing sequences.

In addition, vectors can be engineered to contain a unique protease cleavage sequence downstream of the 5′ end. For example, a protease sequence such as the thrombin cleavage sequence could be placed such that cleavage will produce an active, truncated TNFα-con.

5.3. TRANSFORMATION/TRANSFECTION OF HOST CELLS AND TNFα-CON EXPRESSION

The recombinant expression vector comprising a DNA sequence encoding TNFα-con is preferably transformed or transfected into one or more cells of a substantially homogeneous culture of a suitable host microorganism or insect or mammalian cell line. The expression vector may be introduced into the host cell in accordance with known techniques, including but not limited to transformation using calcium phosphate-precipitated DNA, microinjection of DNA, electroporation, transfection by contacting the cells with a virus, liposome-mediated transfection, DEAE-dextran transfection, transduction, conjugation, microprojectile bombardment, *etc.*

Once the expression vector is introduced into the host cell, the integration and maintenance of the polypeptide coding sequence into the host cell genome, or episomally, can be confirmed by standard techniques, *e.g.*, by Southern hybridization analysis, PCR analysis, including reverse transcriptase-PCR (RT-PCR), or by immunological assays for the expected protein products.

Host cells containing the recombinant TNFα-con coding sequence and that express biologically active product may be identified by at least four general approaches: (i) DNA-DNA, DNA-RNA or RNA-antisense RNA hybridization; (ii) detecting the presence or absence of "marker" gene functions; (iii) assessing the level of transcription as measured by the expression of TNFα-con mRNA transcripts in the host cell; and (iv) detecting the

presence of mature gene product as measured, for example, by immunoassay or by the presence of biological activity.

In the first approach, the presence of the TNFα-con DNA sequence can be detected by nucleotide hybridization using labeled probes that are homologous to the TNFα-con coding sequence.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions, *e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, *etc.* For example, if the TNFα-con coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the TNFα-con coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the TNFα-con sequence under the control of the same or different promoter used to control the expression of the TNFα-con coding sequence. Expression of the marker in response to induction or selection indicates expression of the TNFα-con coding sequence. For example, but not by way of limitation, expression of the FLAG™ epitope, the coding sequence of which can be placed in tandem with the TNFα-con sequence as described above, is detectable in cell extracts using anti-FLAG M2 monoclonal antibodies (IBI) in conjunction, for example, with the Western Exposure™ chemi-luminescent detection system (Clontech).

In the third approach, transcriptional activity of the TNFα-con coding region can be assessed by hybridization assays. For example, total cellular mRNA can be isolated and analyzed by Northern blot using a probe that is homologous to the TNFα-con coding sequence or to particular portions thereof.

In the fourth approach, the expression of the mature protein product can be assessed immunologically,

as for example by Western blots, radio-immunoprecipitation, enzyme-linked immunoassays and the like. Alternatively, protein expression can be confirmed and further characterized by histochemical localization using known methods. See, for example, Bullock and Petrusz (eds), 1982, Techniques in Immunocytochemistry, Vol I, Academic Press, Inc., London, which is incorporated herein by reference. For example, but not by way of limitation, cells or tissues transformed with an expression vector of the invention can be sectioned, and the sections probed with either polyclonal or monoclonal primary antibodies raised against the polypeptide. Bound primary antibodies may then be detected by standard techniques, *e.g.*, using the biotinylated protein A-alkaline phosphatase-conjugated streptavidin technique, or a secondary antibody bearing a detectable label that binds to the primary antibody.

An important test of the success of the expression system involves the detection of TNFα-con exhibiting a biological activity. One of the biological activities associated with TNFα-con protein is its ability to enzymatically convert TNFα precursor to mature TNFα. Another is the ability of TNFα-con to cleave a synthetic substrate. Accordingly, non-limiting methods for detecting the presence of biologically active TNFα-con include detecting the conversion of TNFα precursor to mature TNFα or the cleavage of a synthetic substrate. For example, but not by way of limitation, TNFα-con biological activity can be followed by chromatographically detecting cleavage of a synthetic substrate such as, for example, DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH_2 (SEQ ID NO 14) (DNP = dinitrophenylalanine), using high performance liquid chromatography (HPLC). This synthetic substrate spans the cleavage site of human TNFα precursor. Enzyme activity may be assayed by incubating an enzyme preparation with synthetic substrate (50 μM) in a buffer

such as 0.25 M sucrose and 10 mM HEPES, pH 7.5; or in 50 mM HEPES, pH 7.5, 150 mM KCl, 5 μM $ZnSO_4$, and 2 mM $CaCl_2$ containing DNP-Ser (20 μM) (used as a standard to correct for injection errors), in a final volume of 100 μl. The buffer may also contain the following protease inhibitors: leupeptin (10 μM), pepstatin (1 μM), phosphoramidon (10 μM), AEBSF (1 mM) and E-64 (10 μM) (Sigma or Calbiochem). The assay is preferably carried out at 37°C for 15 min to 3 hr. The reaction may then be quenched by addition of an equal volume of 1% heptafluorobutyric acid (HFBA). To distinguish between general proteolytic activity and activity due specifically to TNFα-con, duplicate samples are run containing a hydroxamic acid-related inhibitor, such as GI 129471, which is a specific inhibitor of TNFα-con (McGeehan *et al.*, 1994, above). Analysis of proteolytic activity may be carried out by separating and detecting substrate and products, for example, by HPLC on a C-18 Vyadac column using a water/acetonitrile gradient from 22 to 35% acetonitrile, with both the water and acetonitrile containing 0.1% HFBA.

Alternatively, TNFα-con activity may be detected by tracking a fluorescent tag after cleavage of a synthetic substrate, such as Z-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Lys(X)-Ser-Arg (SEQ ID NO 17), where Z is a fluorophore such as NBD (6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) or rhodamine, and X, which is linked as a side group to Lys, is, for example, dimethyl coumarin (DMC).

Alternatively, TNFα-con activity may be detected using a radiolabelled precursor. For example, TNFα precursor polypeptide may be radiolabelled by incorporation of ^{35}S-cysteine using an *in vitro* transcription/translation kit (Promega). The radiolabelled substrate is preferably incubated for 1-3 hr at 37°C with the TNFα-con preparation in a buffer containing 0.1-1.0% NP-40, 0.25M sucrose, 10 mM HEPES, pH

7.5, and protease inhibitors (10 μM leupeptin, 10 μM phosphoramidon, 1 mM AEBSF, 10 μM E-64, 1 μM pepstatin, 10 μM diprotinin A, 10 μM amstatin, 10 μM bestatin and 10 μM diprotinin B). The reaction may be quenched by adding loading buffer containing 4% SDS, 200 mM dithiothreitol, 20% glycerol, and 0.2% bromophenol blue. Samples are boiled, loaded onto polyacrylamide gels to separate substrate from product, and visualized using a phosphorimager (Molecular Dynamics, model 425F).

Alternatively, TNFα-con activity may be determined indirectly using an *in vitro* bioassay that detects the presence of TNFα. Any one of several bioassays known in the art can be used, such as a cytotoxicity assay using L-929 murine fibroblast cells (Matthews and Neale, 1987, above).

5.4. PURIFICATION AND CHARACTERIZATION OF EXPRESSED TNFα-CON

Once the structural gene has been stably introduced into appropriate host cells, the host cells may be grown under conditions conducive to maximum production of a biologically active TNFα-con. Such conditions will typically include growing cells to high density. Where the expression vector comprises an inducible promoter, induction conditions may be employed such as, for example, temperature change, exhaustion of nutrients, accumulation of excess metabolic by-products, or the like, as appropriate to induce expression.

Where the expressed protein is retained in the host cells, the cells are harvested, lysed and the product isolated and purified from the lysate under extraction conditions known in the art to minimize protein degradation, such as, for example, at 4°C, or with protease inhibitors, or both. Where the expressed protein is secreted, the exhausted nutrient medium may simply be collected and the product isolated therefrom.

The expressed protein may be purified using standard methods, including but not limited to any combination of the following methods: ammonium sulfate precipitation, size fractionation, ion exchange chromatography, *e.g.*, on a DEAE-cellulose column, HPLC, density centrifugation, and affinity chromatography. TNFα-con may be affinity-purified by binding the polypeptide to: (a) a monoclonal antibody raised against the polypeptide; (b) a lectin, such as conA; (c) TNFα or its precursor; or (d) a TNFα-con inhibitor, for example, a hydroxamic acid-related inhibitor such as GI 129471 (see McGeehan *et al.*, 1994, above), among others. For example, but not by way of limitation, TNFα-con can be affinity-purified using a biotinylated hydroxamic acid-related inhibitor prepared as described below (see Section 7). Briefly, a cell lysate, exhausted culture medium or partially purified enzyme preparation comprising TNFα-con may be contacted with a biotinylated TNFα-con inhibitor under conditions conducive to binding of TNFα-con to the biotinylated inhibitor to form a TNFα-con-inhibitor-biotin conjugate. The TNFα-con-inhibitor-biotin conjugate may then be isolated by contacting it with streptavidin bound to a solid phase matrix, such as ULTRALINK™ Immobilized Neutravidin Plus on 3M EMPHAZE™ Biosupport Medium ABI (Pierce), under conditions conducive to binding of the TNFα-con-inhibitor-biotin conjugate to the streptavidin. The enzyme may then be eluted, for example, by incubation of the support medium overnight in a low salt buffer such as 10 mM NaCl, with protease inhibitors, such as leupeptin (10 μM), pepstatin (1 μM), phosphoramidon (10 μM), AEBSF (1 mM) and E-64 (10 μM).

Increasing purity of the enzyme preparation can be monitored at each step of the purification procedure by known methods, such as by determining protein yield versus enzymatic activity after each successive

purification step. Purity can be assessed by electrophoretic or chromatographic techniques.

Once a TNFα-con polypeptide of sufficient purity is obtained, it may be characterized by standard methods, such as by determining its enzyme kinetics and substrate specificity. For example, the ability of a purified TNFα-con to cleave short peptides may be determined. These peptides will preferably span the cleavage sequence of TNFα precursor, but may be modified, for example, by the presence of amino acid substitutions, deletions or derivatizations, or by differences in substrate length (see Section 8, below).

The amino acid sequence of the TNFα-con protein can be deduced from the cDNA sequence or, alternatively, by direct sequencing of the protein, *e.g.*, with an automated amino acid sequencer. The deduced amino acid sequence of human TNFα-con (SEQ ID NO 2) is depicted in FIG 1.

The TNFα-con protein sequence can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. *USA* 78:3824), which can be used to identify hydrophobic and hydrophilic regions of the TNFα-con protein and, accordingly, the corresponding regions of the gene sequence which encode such regions.

Secondary structural analysis (Chou and Fasman, 1974, Biochem. 13:222) can be carried out to identify regions of TNFα-con that assume specific secondary structures. Biophysical methods such as X-ray crystallography (Engstrom, 1974, Biochem. Exp. Biol. 11:7-13), computer modeling (Fletterick and Zoller (eds) 1986, in: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), and nuclear magnetic resonance (NMR) may be used to map and study sites of interaction between TNFα-con and its substrate. Once these sites have been identified, the present invention provides means for promoting or

inhibiting this interaction, depending upon the desired biological outcome. Based on the foregoing, given the physical information on the sites of interaction, compounds that modulate TNFα-con activity may be elaborated by standard methods known in the field of rational drug design. In addition, methods known in the art, including enzyme digestion of glycolytic side chains, lectin binding, and NMR structural analysis, allow for analysis of the glycosylation pattern of the expressed versus the naturally occurring enzyme.

The present invention includes other methods for identifying the specific site(s) on the TNFα-con polypeptide that interact with TNFα precursor. For example, site-directed mutagenesis of DNA encoding the TNFα-con protein may be used to destroy, inhibit or otherwise alter the interaction between TNFα-con and TNFα precursor, thus producing variants such as TNFα-con antagonists.

In an embodiment of the invention, a series of deletion mutants in the TNFα-con coding region may be constructed and analyzed to determine the minimum amino acid sequence requirements for binding to and proteolytically cleaving TNFα precursor or a synthetic substrate. Deletion mutants of the TNFα-con coding sequence may be constructed using methods known in the art which include but are not limited to use of nucleases and/or restriction enzymes, site-directed mutagenesis techniques, *etc.* The mutated polypeptides may be assayed for their ability to bind to the TNFα precursor or to a synthetic substrate, for example, by gel filtration assays.

In addition, derivatives, analogs and peptides related to TNFα-con can be chemically synthesized (Merrifield, 1985, Science 232:341-347). For example, a peptide corresponding to a portion of TNFα-con that exhibits a desired biological activity can be made using a peptide synthesizer.

5.5. DERIVATIVES, ANALOGS AND PEPTIDES OF TNFα-CON

The production and use of derivatives, analogs and peptides related to TNFα-con are also within the scope of the invention and can be used, for example, in immunoassays, for immunizations, therapeutically, *etc.* Such molecules which retain, inhibit, or otherwise modulate a desired TNFα-con biological activity property can be used as agonists, antagonists, inhibitors or, more generally, as modulators of such an activity. The terms "agonist", "antagonist", "inhibitor" and "modulator" are used in a functional sense and are not intended to limit the invention to compounds having a particular mode of action. Derivatives, analogs and peptides related to TNFα-con can be tested for the desired biological activity by procedures such as those described above, including but not limited to detecting substrate binding and/or cleavage, or by use of one or more *in vitro* TNFα bioassays.

The derivatives, analogs and peptides of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur either at the gene or protein level, or both. At the gene level, for example, the cloned TNFα-con DNA sequence can be modified *in vitro* by any of numerous strategies known in the art. See Maniatis, *et al.*, 1989, above; Ausubel *et al.*, 1989, above; and Sambrook *et al.*, 1989, above. Such modifications include but are not limited to endonuclease digestion, mutations to create or destroy translation, initiation, and/or termination sequences, or that create variations in the coding region, or any combination thereof. Any technique for mutagenesis known in the art can be used, including but not limited to *in vitro* site-directed mutagenesis (see, for example, Hutchinson *et al.*, 1978, J. Biol. Chem. 253:6551).

As a result of alterations at the gene level, the expressed polypeptide may contain deletions,

additions or substitutions of amino acids which may or may not result in a silent change within the sequence to produce a biologically active variant.

Manipulation of the TNFα-con DNA sequence may also be made at the protein level. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to: substitution of one or more L-amino acids of the TNFα-con polypeptide with corresponding D-amino acids, amino acid analogs or amino acid mimics, *e.g.*, so as to produce carbazates or tertiary centers; or specific chemical modification, as for example with cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*

An example of a peptide of TNFα-con would be a truncated version of TNFα-con which, for example, may be produced by removal of a transmembrane domain normally present in the native protein, so as to produce a soluble, secreted form of the enzyme, or that comprised only the catalytic domain of the enzyme. Another example would be a 5′ truncation that would remove the putative cystein switch, thereby activating and/or enhancing convertase activity.

The TNFα-con polypeptide, or a peptide or analog thereof, may be derivatized by conjugation to the protein of other chemical groups, including but not limited to acetyl groups, glycosyl groups, lipids, and phosphates, among others. Such conjugation is preferably by covalent linkage at TNFα-con amino acid side chains and/or at the N-terminus or C-terminus of the polypeptide.

Water soluble polymers, especially polyethylene glycol, may be conjugated to TNFα-con to provide additional desirable properties while retaining, at least in part, a desired biological activity, such as TNFα antagonism. These additional desirable properties

include, for example, increased solubility in aqueous solutions, increased stability in storage, reduced immunogenicity, increased resistance to proteolytic degradation, and increased *in vivo* half-life. Water soluble polymers suitable for use with the peptides of the invention include polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, polyvinyl ethyl ethers, and α,β-poly[(2-hydroxyethyl)-DL-aspartamide]. Polyethylene glycol is particularly preferred. Methods of making water-soluble polymer conjugates of proteins are described in, among other places, U.S. Pat. No. 4,179,337; U.S. Pat. No. 4,609,546; U.S. Pat. No. 4,261,973; U.S. Pat. No. 4,055,635; U.S. Pat. No. 3,960,830; U.S. Pat. No. 4,415,665; U.S. Pat. No. 4,412,989; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,414,147; U.S. Pat. No. 3,788,948; U.S. Pat. No. 4,732,863; U.S. Pat. No. 4,745,180; EP No. 152,847; EP No. 98,110; and JP No. 5,792,435.

Such derivatives, analogs and peptides may be used to compete with full length wild-type TNFα-con protein for binding to TNFα precursor, and in so doing serve to inhibit or antagonize TNFα-con activity. The inhibition of TNFα-con protein function by these antagonists may be useful to reduce TNFα levels in serum or tissues of a mammalian subject, thereby treating septic shock, cachexia or other diseases or conditions characterized by elevated or otherwise abnormal levels of TNFα. Alternatively, TNFα-con derivatives, analogs and peptides that are capable of binding to mature TNFα may be used to neutralize excess levels of TNFα in the serum or tissues of a mammalian subject in need of such treatment.

5.6. SCREENING FOR TNFα-CON INHIBITORS

Recombinantly expressed TNFα-con may be used to screen for molecules that reduce or otherwise modulate TNFα levels by inhibiting or otherwise modulating one or more biological activities of TNFα-con. Such molecules may include small organic or inorganic compounds, antibodies, peptides, or other molecules that inhibit or otherwise modulate the ability of TNFα-con to: (1) bind to TNFα precursor or to a synthetic substrate; (2) convert TNFα precursor to mature TNFα; or (3) cleave a synthetic substrate. Of particular interest are hydroxamic acid-related compounds, several of which have been shown to inhibit TNFα-con activity. See Mohler *et al.*, 1994, above; Gearing *et al.*, 1994, above; McGeehan *et al.*, 1994, above; and WO 95/06031.

Synthetic compounds, natural products, and other potential sources of modulatory compounds can be screened in a number of ways. For example, the ability of a test molecule to inhibit the activity of TNFα-con may be measured using standard biochemical methods such as gel filtration assays to detect an effect on binding, or assays that detect an effect on the cleavage of precursor peptides, or using *in vitro* bioassays that detect the presence of TNFα.

One non-limiting method by which a compound capable of binding to TNFα-con may be isolated and identified comprises: (a) conjugating TNFα-con, or a portion thereof, to a solid phase matrix; (b) contacting the TNFα-con-solid phase matrix conjugate with a material comprising a test compound for an interval and under conditions sufficient to allow the compound to bind to the conjugated enzyme; (c) washing away unbound material from the solid phase matrix; (d) detecting the presence of compound bound to the conjugated TNFα-con; (e) eluting the bound compound from the immobilized enzyme, and (f) collecting and thereby isolating the compound. Alternatively, the compound may first be eluted from the

immobilized enzyme and then detected and characterized. Once isolated, the compound can be tested for its ability to inhibit or otherwise modulate one or more biological activities of TNFα-con.

Random peptide libraries consisting of all possible combinations of amino acids may be used to identify peptides that are able to bind to TNFα-con. Identification of peptides that are able to bind to TNFα-con may be accomplished by screening such a peptide library with recombinant TNFα-con proteins. Alternatively, any binding domains of TNFα-con may be separately expressed and used to screen peptide libraries.

One non-limiting way to identify and isolate a peptide that interacts and forms a complex with TNFα-con involves attaching a detectable label to TNFα-con protein to facilitate the identification of such a complex. Thus, TNFα-con may be conjugated to an enzyme such as alkaline phosphatase or horseradish peroxidase, or to a fluorescent tag, such as fluorescein isothyiocynate (FITC), phycoerythrin (PE) or rhodamine, among others. Conjugation of a detectable label to TNFα-con may be performed using techniques that are routine in the art. Alternatively, TNFα-con expression vectors may be engineered to express a TNFα-con fusion protein containing an epitope for which a commercially available antibody exists, such as the FLAG™ epitope as described above. The epitope-specific antibody may be tagged using methods well known in the art including, for example, by labeling with enzymes, fluorescent dyes or colored or magnetic beads, or the epitope-specific antibody may be detected using a labelled secondary antibody.

A DNA sequence encoding a peptide that interacts with TNFα-con to form a complex may be cloned into an appropriate expression vector for overexpression in either bacteria or eukaryotic cells. The peptide may be purified from cell extracts by known methods.

Alternatively, the peptide may be synthesized by solid phase techniques followed by cleavage from resin and purification by HPLC. Once isolated, the peptide can be tested for its ability to inhibit or otherwise modulate the biological activity of TNFα-con.

5.7. ANTI-TNFα-CON ANTIBODY PRODUCTION

The production of polyclonal and monoclonal antibodies that bind to TNFα-con falls within the scope of the invention. Antibodies to TNFα-con may be useful, for example, as affinity reagents to purify native or recombinant TNFα-con, or to detect the presence of TNFα-con, for example, in histological sections, in cell or tissue extracts, in culture medium, or in enzyme preparations, or therapeutically to neutralize TNFα-con activity.

Either the entire TNFα-con polypeptide or a sub-sequence thereof may be used as immunogen against which antibodies can be raised. For example, the catalytic domain of the enzyme may be isolated and used as an immunogen against which antibodies can be raised.

For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, *etc.*, may be immunized by injection with TNFα-con or a portion thereof. Immunizations are carried out according to known methods. Various adjuvants may be used to increase the immunological response, depending on the host species, including Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum, among others.

Monoclonal antibodies to TNFα-con may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines

in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, Immunology Today, 4:72; Cote *et al.*, 1983, Proc. Natl. Acad. Sci. *USA*, 80:2026-2030) and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" by splicing genes, as for example from a mouse antibody molecule of appropriate antigen specificity, together with genes from a human antibody molecule of appropriate biological structure or activity, can be used (Morrison *et al.*, 1984, Proc. Natl. Acad. Sci. *USA* 81:6851-6855; Neuberger *et al.*, 1984, Nature, 312:604-608; Takeda *et al.*, 1985, Nature, 314:452-454). Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce TNFα-con-specific single chain antibodies.

Antibody fragments which contain specific binding sites for the TNFα-con protein may be generated by known techniques. For example, such fragments include but are not limited to: $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule, and Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, Science, 246:1275-1281) to allow rapid identification of Fab fragments having the desired specificity to the TNFα-con protein.

Techniques for the production of monoclonal antibodies, antibody fragments, *etc.*, are well-known in the art, and are additionally described in Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, which is incorporated herein by reference.

5.8. ANTI-SENSE OLIGONUCLEOTIDES AND RIBOZYMES

Also within the scope of the invention are oligonucleotide sequences that include anti-sense oligonucleotides, phosphorothioates, and ribozymes that function to bind to, degrade and/or inhibit the translation of TNFα-con mRNA.

Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the cDNA sequence encoding TNFα-con (FIG. 1) can be synthesized, for example, by conventional phosphodiester techniques.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of TNFα-con RNA sequences are also within the scope of the invention.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays, for example.

Both the anti-sense oligonucleotides and ribozymes of the invention may be prepared by known methods. These include techniques for chemical synthesis, such as for example by solid phase phosphoamite chemical synthesis. Alternatively, anti-sense RNA molecules may be generated by *in-vitro* or *in-vivo* transcription of DNA sequences encoding the RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters.

Various modifications to the oligonucleotides of the invention may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxyribo-nucleotides to the 5′ and/or 3′ ends of the molecule or the use of phosphorothioate or 2′-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

5.9. METHODS FOR THERAPEUTIC USES

Compounds that inhibit the conversion of TNFα precursor to mature TNFα or otherwise modulate the effective levels of TNFα in the sera or tissues of a mammalian subject can be used to treat diseases or conditions related to elevated or otherwise abnormal levels of TNFα in the subject.

The term "treatment" as used herein with reference to a disease or condition is used broadly and is not limited to a method of curing the disease or condition. The term "treatment" includes any method that serves to reduce one or more of the pathological effects or symptoms of a disease or condition, or to reduce the rate of progression of one or more of such pathological effects or symptoms.

Diseases or conditions that may be treated by the methods of the invention are diseases characterized by one or more of the following criteria: elevated or otherwise abnormal levels of TNFα in serum or tissues of a mammalian subject; the development of septic shock; or the development of cachexia. The terms "elevated" and "abnormal" as used herein are relative terms and are used to describe the levels of TNFα in a subject in need of treatment as compared to a normal subject of similar age, gender, weight, *etc.*

The present invention provides methods for treating such diseases or conditions in a mammalian subject in need of such treatment, comprising administering to that subject an effective amount of a compound that reduces or otherwise modulates the level or biological activity of TNFα in that subject. The compound can alter the absolute or effective amount of TNFα in the subject by any mechanism such as, for example, by inhibiting the ability of TNFα-con to convert TNFα precursor to TNFα, or by inhibiting the cellular secretion of TNFα, or by binding to and thereby reducing the effective concentration of soluble TNFα in the serum or tissues of the subject.

Diseases or conditions that can be treated according to the method of the present invention include systemic inflammatory response syndrome, reperfusion injury, cardiovascular disease, infectious disease, obstetrical or gynecological disorders, inflammatory disease or autoimmunity, allergic or atopic diseases, malignancies, transplants, among others. More specifically, diseases or conditions which may be treated by the method of the present invention include but are not limited to septic shock, cachexia, AIDS, graft-versus-host disease, cerebral malaria, Crohn's disease, diabetes, osteoporosis, restenosis, psoriasis and rheumatoid arthritis, macular degeneration, osteoarthritis, inflammatory bowel disease, and

autoimmune disease such as multiple sclerosis, among others. The method of the present invention may also be used to prevent or reduce the extent of infarction due, for example, to an ischemic event.

The present invention further contemplates the use of combination therapy, wherein one or more compounds that inhibit or otherwise modulate a biological activity of TNFα-con, as disclosed above, can be used in combination with one or more other reagents in the treatment of a disease or condition in a mammalian subject. Such other reagents may include, for example, small organic or inorganic molecules or antibodies directed to TNFα-con or to another component or factor underlying a disease or condition in a mammalian subject, in which combination therapy will serve to increase or otherwise improve the efficacy of treatment of the disease or condition. For example, one or more TNFα-con inhibitors may be used in conjunction with an antibody directed against a component of the inflammatory response such as that involved in an autoimmune disease, such as, for example, rheumatoid arthritis, to increase the efficacy of treatment. For example, one or more TNFα-con inhibitors may be used in conjunction with an anti-CD4 antibody or with an anti-CD23 antibody to treat an autoimmune disease. A humanized anti-CD4 antibody is disclosed in PCT GB 91/01578. Anti-CD23 antibodies are described in Dougall *et al.*, 1994, Tibtech 12:372-379. Alternatively, one or more TNFα-con inhibitors may be used in conjunction with more conventional treatments, such as with methotrexate or cyclosporin A, among others.

The present invention further contemplates a complex comprising a TNFα-con and a therapeutic agent capable of modulating the activity of the enzyme for use in treating a disease or condition associated with TNFα. Such a complex may further comprise the enzyme's substrate.

The present invention further provides pharmaceutical compositions or formulations for use in a method of treatment, comprising one or more compounds that reduce or otherwise modulate the effective level of TNFα in sera or tissues of a mammalian subject and a pharmaceutically acceptable carrier. The invention further encompasses formulations for a combination therapeutic comprising one or more compounds that inhibit the biological activity of TNFα-con, one or more compounds that inhibit some other component involved in a disease or condition in a mammalian subject to be treated, and a pharmaceutically acceptable carrier.

A variety of aqueous carriers may be used in the pharmaceutical formulation of the invention, such as water, buffered water, 0.4% saline, 0.3% glycine, and the like. The pharmaceutical formulations may also comprise additional components that serve to extend the shelf-life of pharmaceutical formulations, including preservatives, protein stabilizers, and the like. The formulations are preferably sterile and free of particulate matter (for injectable forms). These compositions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, *e.g.*, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, *etc.* The formulations of the invention may be adapted for various forms of administration, including orally, intramuscularly, subcutaneously, intravenously, intraocularly, and the like. Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 17th Ed., Mack Publishing

Company, Easton Pa (1985), which is incorporated herein by reference.

The present invention further provides formulations for the sustained release of one or more compounds that reduce or otherwise modulate the total or effective TNFα levels in a subject by inhibiting or otherwise modulating the biological activity of TNFα-con. Examples of such sustained release formulations include composites of biocompatible polymers, such as poly(lactic acid), poly(lactic-co-glycolic acid), methylcellulose, hyaluronic acid, collagen, and the like. The structure, selection and use of degradable polymers in drug delivery vehicles have been reviewed in several publications, including A. Domb *et al.*, 1992, Polymers for Advanced Technologies 3:279-292. Additional guidance in selecting and using polymers in pharmaceutical formulations can be found in the text by M. Chasin and R. Langer (eds.), 1990, "Biodegradable Polymers as Drug Delivery Systems", in: Drugs and the Pharmaceutical Sciences, Vol 45, M. Dekker, New York.

Liposomes may also be used to provide for the sustained release of TNFα-con antagonists or other modulating compounds. Details concerning how to use and make liposomal formulations of drugs of interest can be found in, among other places, U.S. Pat. No 4,944,948; U.S. Pat. No. 5,008,050; U.S. Pat. No. 4,921,706; U.S. Pat. No. 4,927,637; U.S. Pat. No. 4,452,747; U.S. Pat. No. 4,016,100; U.S. Pat. No. 4,311,712; U.S. Pat. No. 4,370,349; U.S. Pat. No. 4,372,949; U.S. Pat. No. 4,529,561; U.S. Pat. No. 5,009,956; U.S. Pat. No. 4,725,442; U.S. Pat. No. 4,737,323; U.S. Pat. No. 4,920,016. Sustained release formulations are of particular interest when it is desirable to provide a high local concentration of a TNFα-con antagonist, for example, at the site of an infection, *etc.*

A purified TNFα-con inhibitor or other modulating compound may be combined with compatible,

nontoxic pharmaceutical excipients and administered to a mammalian subject, *e.g.*, to treat a disease or condition characterized by an elevated or otherwise abnormal level of TNFα in the serum or tissues of the subject. The term "mammalian subject" is intended to include humans and animals. In the case of administration to animals, it may be preferable to incorporate the drug into the animal's feed, possibly in a prepared combination of drug and nutritional material ready for use by the farmer. The compound may be administered orally, rectally, transdermally, by pulmonary infiltration, insufflation or parenterally (including intravenously, subcutaneously and intramuscularly) to humans, in any suitable pharmaceutical dosage form.

An effective dosage and treatment protocol may be determined by conventional means, starting with a low dose in laboratory animals and then increasing the dosage while monitoring the effects, and systematically varying the dosage regimen as well. Numerous factors may be taken into consideration by a clinician when determining an optimal dosage for a given subject. Primary among these is the level of TNFα in serum or tissue of the subject. Additional factors include the size of the subject, the age of the subject, the general condition of the subject, the particular disease or condition being treated, the severity of the disease, the presence of other drugs in the subject, the *in vivo* activity of the antagonist or modulating compound and the like. The trial dosages would preferably be chosen after consideration of the results of animal studies and the clinical literature with respect to administration of modulators of TNFα or TNFα-con. It will be appreciated by the person of ordinary skill in the art that information such as binding constants and K_i derived from *in vitro* TNFα-con binding competition assays may also be used in calculating dosages.

A typical daily human dose of a TNFα-con antagonist or other modulating compound may be in an amount, for example, of from about 0.1 mg to about 200 mg per kilogram of body weight, more preferably from about 1 mg to about 100 mg per kilogram body weight, and most preferably about 5 mg to about 50 mg per kilogram body weight.

5.10. GENE THERAPY

Also within the scope of the present invention is the use of gene therapy to replace mutated TNFα-con with a wild type complement of the gene, or to transfer nucleotide sequences that are anti-sense to a portion of TNFα-con into a subject. Such examples of gene therapy are useful to treat any disease or condition resulting from an elevated or otherwise abnormal level of TNFα or TNFα-con in the subject.

Methods for transferring the wild type TNFα-con gene into the targeted tissue may include reconstitution of recombinant TNFα-con molecules into liposomes for delivery into target cells. Alternatively, recombinant viral vectors may be engineered to express wild type TNFα-con. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes virus or bovine papilloma virus, may be used to deliver wild type TNFα-con into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing TNFα-con coding sequence. See, for example, the techniques described in Maniatis, *et al.*, 1989, above; Ausubel *et al.*, 1989, above; and Sambrook *et al.*, 1989, above.

6. EXAMPLE: ISOLATION OF A cDNA ENCODING HUMAN TNFα-CON

6.1. PURIFICATION OF PORCINE TNFα-CON

6.1.1. MATERIALS AND METHODS

Membrane Preparation

Porcine TNFα-con was isolated from pig spleen. All steps were conducted at 4°C. Approximately 10 to 20 fresh pig spleens, total 3-6 kilograms (kg), were cut into small pieces and placed in a beaker containing cold grinding buffer (10 mM HEPES, pH 7.5, 0.25 M sucrose, 2 mM $MgCl_2$) with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon, and 50 μM DCI). For every volume of spleen tissue, 3 volumes of grinding buffer were used. The pieces were homogenized in a 4L blender using 10 second bursts at low, medium, and then high speed. The ground tissue was passed through a fiberglass screen to filter the suspension. The material passing through the filter was centrifuged in a GS-3 rotor for 10 min at 2000 x g. The supernatant was removed and $CaCl_2$ was added with stirring to a final concentration of 8 mM. Ten minutes after the $CaCl_2$ was dissolved, the solution was centrifuged in a GS-3 rotor for 20 min at 10,000 x g. The pellet was resuspended in 1/6 the volume of the supernatant in buffer (10 mM HEPES, pH 7.5, 0.25 M sucrose) with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon and 50 μM DCI), and this preparation, representing a membrane suspension, was quick frozen and stored at -70°C for future use.

Buffer B (0.05% NP-40, 10 mM HEPES, pH 7.5, 200 mM NaCl) with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon) was added to thawed membrane suspension so that the buffer:membrane volume ratio was 3:1. Three kg of pig spleen produced approximately 1L of membrane

suspension. After mixing the buffer and membrane suspension together, the membranes were pelleted by centrifugation in a TFA 20.250 rotor for 1 hr at 20,000 rpm. The pellet was combined with 6 liters of Buffer C (Buffer B with 1% NP-40) with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon) for every liter of initial membrane suspension. The solution was stirred for 30 min at 4°C followed by centrifugation in the TFA 20.250 rotor for 1 hr at 20,000 rpm.

Chromatography

The supernatant was loaded onto a 1 liter conA column (Pharmacia) equilibrated in the same buffer as the supernatant at a flow rate of 20 ml per min. The column was washed with 5 volumes of Buffer C without protease inhibitors, followed by 5 volumes of Buffer D (same as Buffer C (without protease inhibitors) except that the NaCl concentration was 10 mM. Convertase activity, as determined by HPLC monitoring of the cleavage of a synthetic DNP substrate, was eluted off with 10 column volumes of Buffer E (Buffer D containing 250 mM methyl mannopyranoside) with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin).

The eluant was loaded directly onto a 500 ml Q fast flow column (Pharmacia). The column was washed with Buffer F (0.5% NP-40, and 10 mM HEPES, pH 7.5). Convertase activity was eluted off with Buffer F containing 200 mM or 500 mM NaCl and protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon). The eluted protein was dialyzed against Buffer F without NaCl, but with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin) overnight at 4°C. The material was then loaded onto a 2 ml POROS™ HQ column (Perseptive Biosystems). Elutions were carried out with Buffer F containing 500 mM NaCl and protease inhibitors (1 mM

AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon). Fractions containing convertase activity were further purified by affinity chromatography.

Optionally, before the dialysis step above, the material from the Q-fast flow column was additionally purified with Cibacron Blue 3000 (Sigma). Briefly, the eluant was loaded onto a 300 ml column at a flow rate of 10 ml per min. The column was washed with 3 column volumes of Buffer F containing 200 mM NaCl without protease inhibitors. The activity was then eluted from the column with Buffer F containing 1.5 M NaCl and protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin). The eluted protein was then dialyzed as above.

Affinity Purification

For affinity purification, a biotinylated hydroxamic acid inhibitor, prepared as described below (Section 7), was added to the dialyzed protein to a final concentration of 1 μM. After 10-30 min incubation at 4°C, affinity beads (ULTRALINK™ Immobilized Neutravidin Plus on 3M EMPHAZE™ Biosupport Medium ABI (Pierce)) were added (0.4 ml of slurry per 1 ml of enzyme solution). After incubating for 10 min with gentle rocking, the slurry was pelleted by centrifugation in a microfuge. The beads were washed three times with 1.5 ml of Buffer F containing 0.5 M NaCl. The enzyme was eluted off the beads by gentle rocking overnight in Buffer F containing protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin) without NaCl at 4°C.

Glycerol Gradient

In order to demonstrate that the 85 kDa band co-migrates with convertase activity, the TNFα-con was subjected to a final separation step consisting of sedimentation through a glycerol gradient under

centrifugal force as follows. The material eluted from the affinity resin was layered on top of an 8 to 27.8% glycerol gradient made as follows. The following glycerol dilutions in 10 mM HEPES, pH 7.55 plus 0.05% NP-40 were carefully layered in 12 ml polycarbonate ultracentrifugation tubes (Sorvall, cat. No. 03699): 27.8% (1.1 ml), 25.6% (1.1 ml), 23.4% (1 ml), 21.2% (1 ml), 19% (1 ml), 16.8% (1 ml), 14.6% (1 ml), 12.4% (1 ml), 10.2% (1 ml), 8% (0.8 ml). The tubes were stored at 4°C for 17 hrs to allow for the formation of a continuous gradient, and then 0.2 ml of eluted material was layered on top of each gradient. The gradients were centrifuged at 4°C for 48 hrs at 40,000 rpm in a Sorvall ultracentrifuge (model RC70).

After centrifugation, the gradients were fractionated by taking 0.35 ml aliquots from top to bottom. An aliquot (20 µl) of each fraction was assayed for enzyme activity by detecting cleavage of a synthetic substrate by HPLC as described above. In addition, an aliquot (30 µl) of each fraction was mixed with 10 µl of Laemmli loading buffer (4x stock) and electrophoresed on a 10% polyacrylamide gel under denaturing conditions (Laemmli, 1970, Nature 227:680-685), followed by silver staining (Daichi silver staining kit), in order to correlate the activity of each fraction with proteins bands present in the gel.

Deglycosylation

Reagents for deglycosylation were obtained as a kit from New England Biolabs (Beverly, Mass.). An aliquot (25 µl) of TNFα-con preparation obtained from pooled fractions 16-20 from the glycerol gradient step was incubated for 10 min at 100°C in the presence of 2.5 µl 10x denaturing buffer. The following were then added: NP-40 (3.5 µl); 10x G7 buffer (3.5 µl); and pure PNGase F (0.5 µl; 10 units). The reaction was run for 1 hr at 37°C and then stopped by the addition of 12 µl of 4x

Laemmli loading solution, followed by electrophoresis on a 10% polyacrylamide gel which was then silver stained as described above.

Purification Of Human TNFα-Con

As a means of comparing the molecular mass of human TNFα-con with its porcine counterpart isolated as above, a purification scheme for human TNFα-con was developed based on the above-described procedure. All steps were carried out at 4°C. Thus, a pellet containing 8.2×10^{10} MonoMac6 cells was resuspended to a final volume of 600 ml in grinding buffer. Cells were disrupted by cavitation by exposing them for 30 min to 1,000 psi of nitrogen gas, followed by rapid pressure release. The lysate was centrifuged at 3,500 rpm for 10 min in a GS3 rotor (Sorvall). The supernatant was then centrifuged at 20,000 rpm for 45 min in a TFA20.250 rotor (Sorvall). The pellet was resuspended to 750 ml with buffer C with protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon) containing 1.2% NP-40, and extracted by slow stirring for 30 min, followed by centrifugation for 45 min at 20,000 rpm in the same rotor to remove insoluble material. The supernatant was then loaded on a 100 ml conA-sepharose column at 10 ml/min. The column was washed with 200 ml buffer C without protease inhibitors, followed by 800 ml buffer D with protease inhibitors as above. The activity was then eluted with 850 ml buffer E with protease inhibitors as above. This eluate was applied to a 2 ml POROS™ HQ column washed with 10 ml buffer F, eluted with protease inhibitors, as above, and containing 0.5 M NaCl, and 1 ml fractions were collected. Fractions 2 and 3 which contained over 90% of the TNFα-con activity were pooled. An aliquot (0.5 ml) from this pool was incubated with 1 nmol biotinylated inhibitor as above for 15 min; then 0.1 ml slurry of affinity beads (ULTRALINK™ Immobilized Neutravidin Plus

on 3M EMPHAZE™ Biosupport Medium ABI (Pierce)) was added and incubated for 1 hr, after which the beads were washed three times with buffer F containing 0.5 M NaCl. The activity was eluted from the beads by overnight incubation with buffer F containing 0.1% NP-40 and protease inhibitors (1 mM AEBSF, 1 μM pepstatin, 10 μM E-64, 10 μM leupeptin, 10 μM phosphoramidon) without NaCl at 4°C. The eluted material was further purified on a glycerol gradient as described above. Enzyme activity and SDS-PAGE analysis of human TNFα-con were carried out as described above for porcine TNFα-con. Deglycosylation analysis of human TNFα-con was not carried out because of the limited amount of purified enzyme.

6.1.2. RESULTS

The purity of porcine TNFα-con prepared as above was assessed by SDS-PAGE under reducing conditions (FIG. 2B). Isolated porcine TNFα-con, prepared as above, has an apparent molecular weight of about 85 kDa, as shown by the correlation of enzyme activity with the 85 kDa band throughout the glycerol gradient fractions (FIG. 2A). After deglycosylation, the molecular weight drops to about 62 kDa (FIG. 3). Isolated human TNFα-con has a very similar apparent molecular weight of about 86.5 kDa (Fig. 4B), as shown by the correlation of enzyme activity with the 86.5 kDa band throughout the glycerol gradient fractions (FIG. 4A).

6.2. ISOLATION OF A PARTIAL cDNA SEQUENCE ENCODING PORCINE TNFα-CON

6.2.1. MATERIALS AND METHODS

N-terminal Sequencing

Affinity-purified material was either sequenced directly, or analyzed by SDS-PAGE using two 8-16% Novex (San Diego, CA) mini-gels (100 x 100 x 1mm) in Tris-glycine buffer. Electrophoretically separated proteins were detected using ISS Pro-Green (Natick, MA) according

to manufacturer's directions. A prominent band at about 85 kDa was excised and the protein was electroeluted directly onto a Hewlett-Packard C18 sequencing column, as described by Moyer *et al.*, 1994, in: Crabb, J. (ed), Techniques in Protein Chemistry, Vol. V, pp. 195-204, Academic Press, San Diego, CA. *In situ* reduction, alkylation, and digestion with Lys-con (Wako) were performed according to Burkhart *et al.*, 1993, in: Angeletti, R. (ed), Techniques in Protein Chemistry Vol. IV, pp. 399-406, Academic Press, San Diego, CA, except that 40% acetonitrile was used in the digestion buffer. After digestion, the column was placed in-line on the HPLC using a Hewlett-Packard G1007A column adapter coupled to a Hypersil ODS (0.8 x 300 mm, LC Packings). No peptides were observed following a gradient of 0-80% acetonitrile in 0.1% trifluoroacetic acid (TFA). The sequencing column was subsequently removed from the column adapter and inserted in a Hewlett-Packard G1005S Protein Sequencing System with on-line PTH analysis. A single sequence, as shown below, was obtained after 42 cycles from material bound to the sequencing support.

Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile (SEQ ID NO 7)

Cloning of Porcine TNFα-con

Degenerate and oppositely oriented oligonucleotide PCR primers were designed based on the partial amino acid sequence of porcine TNFα-con determined above, specifically to amino acid sequence Val Gln Asp Val Ile Glu_6 (SEQ ID NO 11) and amino acid sequence Ala Asp Asn Ile Val Gly_{30} (SEQ ID NO 12). Two primers were synthesized from each orientation to reduce the sequence degeneracy. Thus, primer conv-1 has sequence 5'-GTI CA(A/G) GA(T/C) GT(A/G) AT(T/C/A) GA-3'

(SEQ ID NO 3); primer conv-2 has sequence 5′-GTI CA(A/G) GA(T/C) GT(T/C) AT(T/C/A) GA-3′ (SEQ ID NO 4); primer conv-3 has sequence 5′-CC IAC (A/G/T)AT (A/G)TT (A/G)TC (T/C)GC-3′ (SEQ ID NO 5); and primer conv-4 has sequence 5′-CC IAC (A/G/T)AT (A/G)TT (A/G)TC (A/G)GC-3′ (SEQ ID NO 6). Primers conv-1 (SEQ ID NO 3) and conv-2 (SEQ ID NO 4) represent the 5′ nucleotide sequences and the two primers only differ at one base position as shown above. Conv-3 (SEQ ID NO 5) and conv-4 (SEQ ID NO 6) are the 3′ primers as shown.

Reverse transcriptase PCR was performed on porcine spleen poly (A+) RNA according to the manufacturer's protocol (Invitrogen cDNA cycle kit). Each of four PCRs used a pair of primers, one 5′ primer *i.e.*, either conv-1 (SEQ ID NO 3) or conv-2 (SEQ ID NO 4), and one 3′ primer, *i.e.*, either conv-3 (SEQ ID NO 5) or conv-4 (SEQ ID NO 6), at a final concentration of 2 mM each. The PCR mixture was cycled 35 times (one cycle = 45 sec at 94°C, 2 min at 55°C, and 3 min at 72°C), followed by electrophoresis on a 1.2% agarose gel. The expected 89 bp PCR fragment (SEQ ID NO 8) was obtained when primer conv-3 (SEQ ID NO 5) was used with primer conv-1 (SEQ ID NO 3) or conv-2 (SEQ ID NO 4) in the reaction. However, primer conv-4 (SEQ ID NO 6) together with conv-1 (SEQ ID NO 3) or conv-2 (SEQ ID NO 4) gave rise to a fragment of approximately 300 bp. The two PCR fragments thus obtained were made blunt-ended, subcloned into Bluescript II SK at the SmaI site, and subjected to DNA sequencing using the Taq dideoxy terminator method. DNA sequence analysis revealed that the 89 bp fragment (SEQ ID NO. 8) encoded an amino acid sequence (SEQ ID NO 15) identical to the known partial peptide sequence of porcine TNFα-con on which the primers were based, as shown below.

5′ GTG CAG GAC GTC ATC GAG CGG TTC TGG GAG TTC ATT GAC
Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp

AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG GCA GAC
Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp

AAC ATC GTC GG 3′ (SEQ ID NO 8)
Asn Ile Val Gly (SEQ ID NO 15)

However, the 300 bp fragment was highly homologous to a human actin-binding protein (filamin) according to a sequence comparison with the GenBank database, indicating that a non-specific PCR fragment was amplified using primer conv-4 (SEQ ID NO 6).

The 89 bp fragment (SEQ ID NO 8) was used as a probe to screen a porcine spleen cDNA library constructed in λgt10. The probe was labeled by random priming (BRL kit) in the presence of a ^{32}P-dCTP.

A single-stranded oligonucleotide of 55 bp was also synthesized as a probe according to the sequence of the 89 bp fragment. The 55 bp oligomer was located between the 5′ and 3′ PCR primers as shown below:

G CGG TTC TGG GAG TTC ATT GAC AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG (SEQ ID NO 13)

The 55 bp oligomer was labeled by kinase-end labeling using γ^{32}P-ATP. Hybridization of the screening filters was performed in a 40% formamide buffer at 39°C, and the final wash was in 1 x SSC (55 bp oligomer probe) or 0.2 x SSC (89 bp probe) at 46°C.

6.2.2. RESULTS

The initial screening resulted in the isolation of four positive clones out of 2.5 x 10^5 recombinants. These four clones (psC-1, psC-2, psC-3 and psC-5) ranged from 1.1 kb to 2.3 kb in size and were sequenced after subcloning into the EcoRI site of Bluescript II SK. The clone psC-3 was completely sequenced in both directions by subcloning the smaller restriction fragments into Bluescript II SK and

using flanking T3 and T7 sequences as sequencing primers. Sequence comparison showed that the four clones are overlapping. FIG. 5 shows a contiguous mapping of the entire length of 2,414 bases covered by the four clones. Sequence analysis demonstrated that the 2,414 bp domain contained the coding sequence of the known 41 amino acid peptide sequence obtained from purified porcine spleen TNFα-con, but did not contain the coding region for the N-terminus of the porcine TNF-con. The conserved Zn^{2+}-binding motif of metalloproteinases was also lacking.

To obtain further 5′ nucleotide sequence of porcine TNF-con, the 5′ 120 bp EcoRI-PstI fragment of psC-2 clone was isolated and used as a probe to rescreen the porcine spleen cDNA library. Three positive clones were obtained. Sequence comparison indicated that only one clone, psC-8, had the extended 5′ sequence. This clone contained a 50 bp coding sequence 5′ to the psC-2 clone. The 50 bp region encodes the HELGH motif. The total 2,464 bp nucleotide sequence (SEQ ID NO 9) and the deduced amino acid sequence (SEQ ID NO 10), which represents a partial sequence of porcine TNFα-con, are shown in FIG. 6.

6.3. ISOLATION OF A cDNA SEQUENCE ENCODING HUMAN TNFα-CON

6.3.1. MATERIALS AND METHODS

Two λgt10 cDNA libraries constructed either from human leukocyte poly(A+) RNA (Clontech) or human monocyte poly(A+) RNA were used to screen for the full length human TNFα-con cDNA. The 120 bp EcoRI-PstI fragment and its flanking 3′ 690 bp PstI-BamHI fragment from the porcine psC-2 clone were labeled by random priming (BRL kit) and used to screen both libraries. Replicate filters were hybridized in 50% formamide at 42°C, and the final wash was in 0.2 x SSC with 0.1% SDS at 55°C. Of approximately 2.5 x 10^5 clones from each library, six of 14 positive clones (hc7, hc9, hc11, 3′#1, 3′#4, 3′#5), as shown in FIG. 7, were plaque-purified and sequenced.

To obtain the extreme 5′ end of the cDNA, two different procedures were carried out, which produced equivalent results. In a first procedure, a 330 bp EcoRI-EcoRV DNA fragment corresponding to the 5′ end of hc11 (FIG. 7) was labelled by random hexamer primer (Amersham), and used to screen the human monocyte library. Filters were hybridized in 50% formamide at 42°C and washed in 0.2 x SSC at 65°C. Seven positive clones were isolated after screening approximately 2.5 x 10^5 clones. Two of the seven clones containing the longest 5′ extensions, as shown in FIG. 7 (5′#4, 5′#7) were plaque-purified for further sequence analysis.

In a second procedure, a 5′ RACE procedure was conducted using a RACE kit from Gibco/BRL, as described below. See also Frohman *et al.*, 1988, Proc. Natl. Acad. Sci. USA, 85:8998-9002, which is incorporated herein by reference.

First Strand cDNA Synthesis

Synthesis of a first strand was carried out using oligonucleotide primer RACE1 (22-mer) having the following sequence: 5′-CCTAGAGTCAGGCTCACCAACC-3′(SEQ ID NO 32), which is complementary to bp no. 541-520 of TNFα-con (FIG. 1) in sequence with Met start at bp no. 164-166. The following were combined: 1 μl RACE1 Oligonucleotide (2.5 pmoles/μl); 1 μl Poly(A+) RNA from THP1-5A cells treated for 3 hr with TNFα and dibutyryl cyclic AMP (0.84 μg); and 9.5 μl DEPC-treated dH_2O. The mixture was incubated at 70°C for 10 min and then chilled on ice for 1 min.

The following was added to the above mixture: 2.5 μl 10X reaction buffer [200 mM Tris-Hcl (pH 8.4), 500 mM KCl]; 3.0 μl 25 mM $MgCl_2$; 1.0 μl 10 mM dNTPs; 2.5 μl 0.1 M DTT; and 0.5 μl RNAsin (20 U). The solution was mixed and incubated at 42°C for 2 min. SUPERSCRIPT II™ reverse transcriptase (1 μl, 200 U, Gibco/BRL) was added and the mixture was incubated at 42°C for 30 min and then at 70°C for 15 min. RNase H (1 μl, 2 U) was added, and the mixture

incubated at 55°C for 10 min, and then chilled on ice. The cDNA was purified using GLASSMAX™ DNA isolation cartridges according to manufacturers protocol [Gibco/BRL, catalog no. 18374-025]. Samples were partially dried *in vacuo* to bring volume to approximately 30 µl.

TdT Tailing of cDNA

The following were combined in three separate equivalent reactions: 7.5 µl DEPC-treated H_2O; 2.5 µl 10X reaction buffer; 1.5 µl 25 mM $MgCl_2$; 2.5 µl 2 mM DCTP; and 10 µl of cDNA sample. The mixtures were incubated at 94°C for 2 min, then chilled on ice for 1 min, and 1 µl terminal deoxynucleotidyl transferase (10 U/µl) was added. The mixtures were then incubated at 37°C for 10 min, at 70°C for 10 min, and then kept on ice.

PCR of cDNA and Recovery

In each of 15 separate equivalent 50 µl reactions, the following were combined: 30 µl dH_2O; 4.0 µl 10X reaction buffer; 3.0 µl 25 mM $MgCl_2$; 1.0 µl 10 mM dNTPs; 1 µl RACE2B Oligonucleotide (10 pmoles/µl); and 1 µl Anchor Oligo-nucleotide (10 pmoles/µl).

RACE2B oligonucleotide (48-mer) has the following sequence (SEQ ID NO 33):

5′-CACGCGTCGACTAGTTACCATCCACCACCACGACCTTGAAATTTTGTG-3′

MluI_____ complementary to bp no. 467-435 in

SalI_____ sequence with Met start at bp no 164-166

SpeI;

Anchor Oligonucleotide, from Gibco/ BRL, has the following sequence (SEQ ID NO 34):

5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′.

The reaction mixtures were incubated at 80°C for 5 min. Five µl of HOT TUB™ Polymerase (Amersham Inc.) in 1X reaction buffer (1 U/5µl) was added, and the mixtures cycled in a Perkin Elmer 9600 thermal cycler 34 times (1 min at 94°C, 30 sec at 50°C, 2 min at 72°C); and 1 time (1 min at 94°C, 30 sec at 50°C, and 10 min at 72°C). All 15 reaction

mixtures were combined and the DNA was precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 20 μl of dH_2O.

DNA was electrophoresed through a 1.5% agarose gel in 0.5X TBE buffer. DNA migrating as a stained smear from approximately 375-700 bp was collected by electro-elution into a well containing 100 mM ammonium acetate. The DNA was extracted once with phenol/chloroform (1:1), and precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 21.5 μl of Dh_2O.

Restriction and Ligation of cDNA into Plasmid

10X H buffer (2.5 μl) (Boehringer Mannheim) and SpeI (1 μl; 10 U/ml) were added to a DNA sample and incubated at 37°C for 2 hr. DNA was electrophoresed through a 1.5% agarose gel in 0.5X TBE buffer. DNA migrating as a stained smear from approximately 200-800 bp was excised from the gel and recovered using a SPINBIND™ cartridge according to manufacturer's protocol (FMC Corp.), with an additional extraction with phenol/chloroform (1:1) after elution from the cartridge. DNA was precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 7.25 μl of dH_2O.

To the DNA was added pBS-SKII+ plasmid (Invitrogen) (1.0 μl, approx. 25 ng) which had been digested with SpeI and dephosphorylated with calf intestinal phosphatase. The mixture was incubated at 55°C for 2 min. The following was then added: 10X T4 Ligase Buffer (1 μl) (Boehringer Mannheim); 0.5 μl 10 mM ATP; and 0.25 μl T4 Ligase (Boehringer Mannheim) (1 U/μl); and the ligation mixture was incubated at 15°C for 7.5 hr.

Bacterial Transformation And Clone Characterization

DH5α MAX EFFICIENCY™ Cells (Gibco/BRL) (100 μl) were transformed with 1.5 μl of the ligation mixture according to manufacturers protocol. Cells were plated onto 100 mm LB plates containing 50 μg/ml ampicillin, overlayed with 75 μl Bluo-Gal and 10 μl 100 mM IPTG, and incubated overnight at 37°C.

Plasmid DNA from white colonies was isolated by standard methods and analyzed for cDNA inserts by digestion with SpeI and electrophoresis through a 1.0% agarose gel in 0.5X TBE buffer. Clones containing cDNA inserts were sequenced and positive clones were identified by comparison to the 5′-nucleotide sequence of TNFc clone hc11. The DNA sequence (SEQ ID NO 39) of the RACE14 clone is shown in FIG. 11.

Constructing A cDNA Encoding The Entire Open Reading Frame (ORF) Of TNF Convertase

Assembly Of RACE14 and hc-11

RACE14 cDNA (SEQ ID NO 39) (FIG. 11) was cloned into the SpeI site of Bluescript plasmid, pBS-SKII+ with the 3′-end of the cDNA oriented closest to the T7 promoter site on the vector. RACE14/pBS-SKII (3,485 bp) was cut with BglII and HindIII and the ends were dephosphorylated. hc-11 cDNA was cloned into the EcoRI site of Bluescript plasmid pBS-SKII+ with the 3′-end of the cDNA oriented closest to the T7 promoter site on the vector. hc-11/pBS-SKII (4,323 bp) was digested with BglII and HindIII to excise the hc-11 cDNA. The BglII/HindIII digested hc-11 cDNA was ligated into the BglII/HindIII digested RACE14/pBS-SKII. The resulting plasmid (4,677 bp) was designated RACE14/hc11-pBS-SKII.

Assembly Of RACE14/hc-11 With ORF Of hc-7 And hc-9

RACE14/hc11-pBS-SKII was digested with EcoRI and NotI, purified from the excised piece of hc-11 cDNA and dephosphorylated. hc-7 cDNA (FIG. 7) was cloned into the

EcoRI site of Bluescript plasmid, pBS-SKII+ with the 3′-end of the cDNA oriented closest to the T3 promoter site on the vector. hc-7/pBS-SKII (4,813 bp) was cut with EaeI and NcoI. DNA fragments were separated on a 1% agarose, 0.5X TBE gel and the 750 bp EaeI/NcoI fragment of hc-7 was isolated using FMC SPINBIND™ cartridges as above. hc-9 cDNA (FIG. 7) was cloned into the EcoRI site in Bluescript plasmid pBS-SKII+ with the 3′-end of the cDNA oriented closest to the T7 promoter site on the vector. hc-9/pBS-SKII (4,895 bp) was cut with EaeI and EcoRI. DNA fragments were separated on a 0.7% agarose, 0.5X TBE gel and the 1,098 bp EaeI/EcoRI fragment of hc-9 was isolated using FMC SPINBIND™ cartridges as above. EcoRI/NotI digested RACE14/hc11-pBS-SKII was mixed with both the 750 bp EaeI/NcoI fragment of hc-7 and the 1,098 bp EaeI/EcoRI fragment of hc-9 and ligated together with T4 DNA ligase.

PCR Generation Of cDNA Encoding The Entire ORF Of TNFα-Con

The ligation mix (10 μl) of RACE14/hc11-pBS-SKII, 750 bp EaeI/NcoI fragment of hc-7 and the 1,098 bp EaeI/EcoRI fragment of hc-9 was subjected to PCR by combining the following: 1 μl of the 10 μl ligation mixture, 1 μl of the 5′ oligonucleotide primer - TNFC-4 (bp 164-190) (SEQ ID NO. 35) (100 pmoles/ml) (see below); 1 μl of the 3′ oligonucleotide primer - TNFC-3 (bp 2754-2719) (SEQ ID NO. 36) (100 pmoles/ml) (see below); 4.5 μl 10X HOT TUB™ buffer (low magnesium; Amersham); 1 μl 10 mM dNTPs; and 36.5 μl dH_2O.

TNFC-4 oligonucleotide sequence (SEQ ID NO 35) is:

5′-CGGGATCCATGAGGCAGTCTCTCCTATTCCTGACC-3′

BamHI

bp no. 164-190 of TNFα-con cDNA.

TNFC-3 oligonucleotide sequence (SEQ ID NO 36) is:

5′-CAGGAAGTTGCGGCCGCTGACCAGCATCTGCTAAGTCACTTCCCAGTCTTCAC-3′

NotI

bp no. 2754-2719 of TNFα-con cDNA (FIG. 1).

The mixture was incubated at 80°C for 5 min, and 5 μl (1 U) HOT TUB™ Polymerase in 1X buffer (Amersham) was then added. The mixture was cycled in a Perkin Elmer 9600 thermal cycler 25 times (1 min at 94°C, 2 min at 55°C, 2 min at 72°C), and 1 time (1 min at 94°C, 2 min at 55°C, 10 min at 72°C).

Restriction Enzyme Digestion and Ligation of cDNA Into Plasmid

The PCR reaction mixture was extracted once with phenol/chloroform (1:1), and precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, resuspended in 1X NEB3 buffer containing 100 μg/ml BSA, 15 U NotI and 10 U BamHI, and incubated for 3 hr at 37°C. Digested DNA was electrophoresed through a 0.7% agarose gel in 0.5X TBE buffer, and DNA migrating at approximately 2,500 bp was excised from the gel and recovered using a SPINBIND™ cartridge as above, followed by an additional extraction with phenol/chloroform (1:1) after elution from the cartridge. The DNA was precipitated at -20°C with 2 M ammonium acetate and 2 volumes of 100% EtOH. DNA precipitate was collected by centrifugation, rinsed with cold 70% EtOH, dried *in vacuo*, and resuspended in 10 μl of dH_2O.

The following were combined in a first tube: 1.0 μl of pBS-SKII+ plasmid (Invitrogen) (approx. 25 ng) previously digested with NotI and BamHI and dephosphorylated with calf intestinal phosphatase; 1 μl of digested PCR-generated cDNA from immediately above; and 6.25 μl dH_2O.

The following were combined in a second tube: 1 μl of PBS-SKII+ plasmid (Invitrogen) (approx. 25 ng) previously digested with NotI and BamHI and dephosphorylated with calf intestinal phosphatase; 3.0 μl of digested PCR-generated cDNA from immediately above; and 4.25 μl Dh_2O.

The mixture in each tube was incubated at 55°C for 2 min. To each tube was added: 1 µl of 10X T4 Ligase Buffer (Boehringer Mannheim); 0.5 µl 10 mM ATP; and 0.25 µl T4 Ligase (Boehringer Mannheim) (1 U/µl), and each tube was incubated at 15°C for 6 hr.

Bacterial Transformation And Clone Characterization

DH5α MAX EFFICIENCY™ cells (Gibco/BRL) (67 µl) were transformed with 1.5 µl each of the ligation mixtures from above according to manufacturers protocol. Cells were plated onto LB plates containing 50 µg/ml ampicillin, overlayed with 75 µl Bluo-Gal and 10 µl 100 mM IPTG, and incubated overnight at 37°C. Plasmid DNA from "white" colonies was isolated by standard methods and analyzed for cDNA inserts by digestion with PstI, and duplicate aliquots were digested with BamHI and NcoI and electrophoresed through a 1.0% agarose gel in 0.5X TBE buffer. Four of 6 clones contained cDNA with the expected restriction enzyme digestion pattern. DNA from 2 clones was sequenced. DNA from one of the two clones, pBS/TNFC-1, was correctly assembled as determined by its DNA sequence. pBS/TNFC-1 corresponds to bp 164-2754 of the cDNA sequence encoding TNFα-con shown in FIG. 1, but with BamHI and NotI ends added to the sequence.

Expression Vector Construction

The full-length cDNA encoding the human TNFα-con was subcloned into a baculovirus expression vector, pFastBac1 (Gibco/BRL), as follows: pBS/TNFC-1 (5 µg) was digested with 1 µl of BamHI (Promega, 10 U/µl); 1 µl of NotI (Promega, 10 U/µl); 1 µl of PvuI (Gibco/BRL, 10 U/µl) (note: PvuI was added to further cut pBluescriptSK and facilitate band identification and isolation from gel); 3 µl of 10x New England Biolabs (NEB) restriction buffer no. 3; and adjusted to a final volume of 30 µl with water. The mixture was incubated for 2 hr at 37°C and then run on a 1% agarose-TAE gel.

The approx. 2.9 kb BamHI - NotI insert band on the gel was excised in a gel piece. The gel piece was frozen at -70°C for 15 min, incubated at 37°C for 15 min, placed inside the upper chamber of a Millipore spin filter unit (ULTRAFREE ™ Probind), and then centrifuged in a microcentrifuge (Eppendorf) for 10 min at maximum speed. The eluate collected at the bottom tube was saved. This material was ligated to pFastBac1 containing the polyhedrin promoter (pFBPH), opened at the multiple cloning site by double digestion with BamHI and NotI, and purified as described above for pBS/TNFC-1. Several ligation reactions were set up at a fixed concentration (50 ng) of pFastBac1 and variable amounts of insert (0, 50 and 250 ng), in a final volume of 20 µl containing 2 µl of 10x T4 DNA ligase buffer and 2 µl of T4 DNA ligase. The reaction was incubated at 12°C overnight.

Ligated material (10 µl) was used to transform 100 µl of DH5α MAX EFFICIENCY™ competent cells (Gibco/BRL) by calcium chloride precipitation according to supplier's instructions. Transformation mixture (100 µl) was plated onto a 2x YT/agar plate containing 100 µg/ml ampicillin. The plate was incubated overnight at 37°C. Colonies were picked up from the plate and diluted in 2 ml of the same medium without agar. These cultures were incubated at 37°C overnight with vigorous shaking. Plasmids were isolated using the WIZARD™ miniprep DNA purification kit (Promega), according to manufacturer's instructions. 5 µl of each isolated plasmid were incubated with 1 µl of 10X NEB buffer no. 4, 0.5 µl of NotI (Promega; 10 U/µl), and 0.5 µl of BamHI (Promega; 10 U/µl), and adjusted to a final volume of 10 µl with water. After 1 hr of incubation at 37°C, samples were run in a 1% agarose-TAE gel, and one candidate with the correct restriction pattern was identified. A larger plasmid preparation was made out of this isolate (100 ml in the same culture medium as described above), and the plasmid was extracted and purified using the Qiagen plasmid Midi kit, according to manufacturer's directions. Sequence of

the pure plasmid was confirmed by sequencing analysis at the Glaxo Wellcome Core DNA Sequencing Facility. This plasmid was designated pFBPH/TNFC.

An expression vector containing a partial TNFα-con cDNA was also constructed in pFastBac, starting at codon 164 (Met) and ending at codon 651 (Arg). This region encodes a polypeptide that spans from before the catalytic domain to before the transmembrane domain. This construct was made as follows. The oligonucleotides M3 and M7 were used to obtain the desired cDNA fragment from PBS/hc-7 by PCR:

M3: 5′-GCGCGCGCGCCATATGTTAGTTTATAAATCTGAAGATATCAAGAAT-GTTTCACG-3′ (SEQ ID NO. 37);

M7: 5′-CGCGCGCGCGGGATCCCTATCGTTCAATTACATCCTGTAC-TCGTTTCTCAC-3′ (SEQ ID NO. 38).

M3 and M7 were diluted after purification to a final concentration of 20 μM in water. Each primer (1 μl) was added to a tube containing 11.5 μl of water, followed by the addition of 2 μl of Stratagene OPTIPREP™ buffer no. 3, 2 μl of 10 mg/ml bovine serum albumin, 1 μl (0.1 μg) of pBS/hc-7, 0.5 μl of a 100 mM deoxynucleotides mixture (Stratagene), and 1 μl of VENT™ polymerase (New England Biolabs).

The reaction mixture was cycled in a Perkin-Elmer model 9600 thermal cycler using the following PCR protocol for a total of 30 cycles: 30 sec at 94°C, 30 sec at 50°C, and 2 min at 72°C. After reaction completion, 2 μl were run on a 1% agarose-TAE buffer to confirm product size and quality. The remaining material was diluted to 85 μl with water, followed by the addition of 10 μl of 10x NEB buffer no. 4 and 5 μl of BamHI (New England Biolabs, 20 U/μl). The restriction reaction was incubated overnight at 37°C, and the sample was resolved in a 1% agarose-TAE preparative gel. The DNA band corresponding to the restricted PCR product was excised, and the gel piece was purified by spin filtration as described above.

The eluate collected from the bottom of the centrifuge tube was used in a ligation reaction to Pfastbac1

(Gibco/BRL) containing an inverted multiple cloning site cut with StuI and BamHI as follows: 10 μl (1 μg) of PfastBac1 were mixed with 5 μl of 10x NEB buffer no. 4, 2.5 μl of Stu I (New England Biolabs, 10 U/μl), and 30 μl of water. The StuI cleavage reaction was allowed to proceed for 3 hr at 37°C, and then 2.5 μl of BamHI (New England Biolabs, 20 U/μl) were added with further incubation at 37°C for 3 hr. The restricted plasmid was purified as described for the PCR product. Ligations were done in a final volume of 20 μl, and transformation and colony screening were done as described above. The ligation reaction consisted of 2 μl (approx. 50 ng) of restricted Pfastbac1, 10 μl (approx. 150 ng) of restricted PCR product, 2 μl of 10x T4 DNA ligase buffer (Promega), and 2 μl of T4 DNA ligase-HC (Promega). The reaction was incubated at 12°C overnight.

Ligated material (10 μl) was used to transform 100 μl of DH5α MAX EFFICIENCY™ competent cells (Gibco/BRL) by calcium chloride precipitation following the supplier's instructions. 100 μl of the transformation mixture were plated onto a 2x YT/agar plate containing 100 μg/ml ampicillin. The plate was incubated overnight at 37°C. Colonies were picked up and DNA was extracted and purified as described above. 5 μl of each isolated plasmid were incubated with 1 μl of 10X NEB buffer no. 4, 0.5 μl of NdeI (New England Biolabs, 10 U/μl), and 0.5 μl of BamHI (Promega, 10 U/μl), and adjusted to a final volume of 10 μl with water. After 1 hr of incubation at 37°C, samples were run in a 1% agarose-TBE gel, and one candidate with the correct restriction pattern was identified. More plasmid was prepared as described above. Sequence of pure plasmid (pFBN2) was confirmed by DNA sequencing analysis.

After obtaining pFBN2 it was determined that pBS/hc-7 contains a T to C base change at position 1,512 (FIG. 1B), resulting in a Phe to Ser change in the translated amino acid sequence. This mutation was fixed by replacing the NcoI to XhoI fragment of pFBN2 (containing the mutation) with a NcoI to XhoI fragment from clone hc-11

(wild type T at position 1,512), in the multi-step procedure described below.

In the first step of the procedure, an additional XhoI site in pFBN2's multiple cloning site was outcloned. 2 µl (4 µg) of pFBN2 were incubated at 37°C for 3 hr with 2 µl of NEB buffer no. 4, 1 µl of NotI (Promega, 10 U/µl), 1 µl of HindIII (Promega, 10 U/µl), and 14 µl of water. T4 DNA polymerase (0.5 µl) and 0.5 µl of a dNTPs mixture (Stratagene, 100 mM each) were then added, and incubation proceeded at 37°C for 1 hr. 2 µl of 10X T4 DNA ligase buffer (Promega) and 2 µl of T4 DNA ligase (Promega, HC) were added, and the mixture was incubated at 12°C for 6 hr. 200 µl of *E. coli* Stbl-2 MAX EFFICIENCY™ competent cells (Gibco/BRL) were added to the tube, and the transformation mixture was incubated on ice for 30 min, followed by a 30 sec pulse at 42°C and plating of the whole mixture on a 2x YT/agar plate containing 100 µg/ml ampicillin. The plate was incubated for 24 hr, and colonies were picked and plasmid minipreps prepared as described above. Restriction analysis was carried out using XhoI, HinDIII, NotI and XhoI-AccI combined in order to confirm the loss of the HinDIII, NotI and the extra XhoI site. One construct with the correct restriction patterns was identified, its plasmid DNA prepared as described before, and sequenced. This plasmid was designated pFBN2/ΔXhoI, and its sequence is identical to pFBN2, except for the deletion of the HinDIII to NotI segment of the multiple cloning site.

In the second step of the procedure, both pFBN2/ΔXhoI and pBS/hc-11 were digested with NcoI and XhoI as follows. 10 µg of either plasmid was mixed with 6 µl of NEB buffer no. 4, 3 µl of NcoI (Promega, 10 U/µl), 3 µl of XhoI (Gibco/BRL, 10 U/µl), and adjusted to a final volume of 60 µl with water. These mixtures were then incubated for 2 hr at 37°C, and loaded on a 1% agarose-TAE preparative gel. The backbone fragment of pFBN2/ΔXhoI and the approx. 380 bp fragment from PBS/hc-11 were excised and purified by spin-filtration as described above. 50 ng (4 µl) of the backbone

fragment were mixed with 12 ng (12 µl) of the small hc-11 fragment, 2 µl of 10x T4 DNA ligase buffer (Promega) and 2 µl of T4 DNA ligase (Promega, HC), and incubated at 12°C overnight. Stbl-2 MAX EFFICIENCY™ competent cells (200 µl; Gibco/BRL) were added, and the mixture was incubated at 0°C for 30 min, followed by a 30 sec pulse at 42°C and plating onto a 2x YT-agar plate containing 100 µg/ml ampicillin. The plate was incubated at 30°C for 24 hr. Colonies were picked and plasmid minipreps were performed as described above. Isolated plasmids were analyzed by double digestion with XhoI (Gibco/BRL, 10 U/µl) and AccI (New England Biolabs, 10 U/µl). One plasmid exhibited the correct restriction pattern, and the corresponding isolate was used for making a larger plasmid preparation as described above. The plasmid thus obtained was sequenced in order to confirm the wild type sequence. This plasmid was designated pFBN2/hc-11, and its sequence is identical to that of pFBN2/ΔXhoI, except for the presence of the wild type T residue at position 1,512.

In the third step of the procedure, pFBN2/hc-11 was used to repair pFBPH/TNFC. Both pFBN2/hc-11 (10 µg) and pFBPH/TNFC (5 µg) were mixed with 5 µl of NEB buffer no. 4, 2.5 µl of NsiI (New England Biolabs, 10 U/µl), 2.5 µl of NcoI (Promega, 10 U/µl), and adjusted to 50 µl with water. Mixtures were incubated for 2 hr at 37°C, and the digests were resolved in a 1% agarose-TAE preparative gel. The backbone fragment of pFBPH/TNFC and the approx. 600 bp fragment from pFBN2/hc-11 were excised and purified as described above. 50 ng (2 µl) of pFBPH/TNFC backbone fragment were then mixed with 50 ng (10 µl) of pFBN2/hc-11, 2 µl of 10x T4 DNA ligase buffer (Promega), 2 µl of T4 DNA ligase (Promega, HC) and 4 µl of water. The reaction was incubated overnight at 12°C, and then 200 µl of Stbl-2 competent cells were transformed with this ligate as described above. Transformant colonies were isolated and the DNA purified and analyzed as described above by performing restriction digests with NcoI and NsiI. Three

correct isolates were worked up as described for sequence confirmation. The new full-length expression vector was designated pFBPH/TNFCA, and differs from pFBPH/TNFC by the presence of the correct T residue at position 1512.

6.3.2. RESULTS

Human TNFα-con is characterized by the cDNA sequence (SEQ ID NO 1) and deduced amino acid sequence (SEQ ID NO 2) shown in FIG. 1. The cDNA contains a 163 bp untranslated region at the 5′ end, followed by a protein coding region of 2,475 bp, and a 304 bp untranslated region at the 3′ end. The first methionine residue (presented as the first amino acid residue in FIG. 1) is the initiation methionine, as indicated by the presence upstream of this residue of a termination codon (TAG) at bases 62 to 64.

Full length TNFα-con comprises 824 amino acids. TNFα-con begins with amino acid Met and ends with amino acid Cys. Based on the deduced amino acid sequence, the predicted molecular weight of the protein is 93.02 kDa. Hydropathy plots reveal 2 hydrophobic segments representing a putative signal peptide at amino acid residues 1-17, and a transmembrane region at amino acid residues 672-691. A search of GeneBank identified the following motifs that are unique to the metalloproteinase family: cysteine switch motif (aa 181-185), zinc-binding motif (aa 405-409), and Met-turn motif (aa 435-437).

7. EXAMPLE: PREPARATION OF A BIOTINYLATED INHIBITOR OF TNFα-CON

A biotinylated inhibitor of TNFα-con, useful for the affinity purification of TNFα-con as described above (Section 6.1.1), was prepared as follows.

Unless otherwise noted, chemicals were obtained from commercial suppliers and used without further purification. Thin layer chromatography (TLC) analyses were performed with Merck 60 F_{254} 0.25 micron silica gel plates.

Flash column chromatography was performed using 230-400 mesh silica gel (EM Science). Compound homogeneity was determined by analytical reverse-phase HPLC using a Dynamax-60A column with eluants A (water, 0.1% TFA) and B (acetonitrile, 0.1% TFA) with gradient elution from 85% A:15% B to 20% A:80% B over 30 min with a flow rate of 1.5 ml per min. Compound purification was carried out by preparative reverse-phase HPLC using a 2-in. diameter Dynamax-60A column using the same eluants as described for analytical HPLC with an appropriately varied gradient at a flow rate of 45 ml per min. ^{1}H NMR spectra were obtained with a Varian Unity 300 with line broadening of 0.5 Hz and a relaxation delay of 0.1 seconds. Proton-decoupled ^{13}C spectra were obtained at 75 MHz using the same instrument. Chemical shifts are reported in ppm. Low resolution mass spectra were performed on a JEOL AX505 using fast atom bombardment (FAB) using thioglycerol, 3-nitrobenzyl alcohol, or 3-nitrobenzyl alcohol/lithium acetate as the matrix solvent.

Preparation of (A)

To a solution of CBZ-phenylalanine (5.57 g, 0.0192 mol) in CH_2Cl_2 (70 ml) at 25°C were added HOBt•H_2O (2.94 g, 0.0192 mol), BOC-1,6-hexanediamine (5.0 g, 0.0198 mol) and Et_3N (2.97 ml, 0.0213 mol). The solution was cooled to 0°C. To the solution was added dicyclohexylcarbodiimide (4.31g, 0.0209 mol), and the solution was stirred and allowed to warm to 25°C over 16 hr. The mixture was filtered, the filtrate was washed using dilute HCl, and the layers were separated. The organic layer was washed using saturated, aqueous $NaHCO_3$ and the layers were separated. The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated to afford 9.53 g (99%) of product (A) (FIG. 9A). This material was used in the next reaction without further purification. Rf 0.42 (30% EtOAc:CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00 - 1.40 (m, 8H), 1.42 (s, 9H), 2.9 - 3.2 (m, 6H), 4.35 (q, 1H, J = 7 Hz), 4.55 (br s, 1H), 5.08

(s, 2H), 5.44 (br s, 1H), 5.78 (br s, 1H), 7.10 - 7.40 (m, 10H).

Preparation of (B)

To a solution of product (A) (5.00 g, 0.0100 mol) in EtOH (100 ml), H_2O (1.0 ml), and EtOAc (40 ml) was added 10% Pd/C (1.00 g) and the reaction flask was evacuated and purged using nitrogen. The reaction flask was evacuated and purged using hydrogen and the mixture was stirred at 25°C under 1 atm of hydrogen for 6 hr. The reaction flask was evacuated and purged using nitrogen, the mixture was filtered, and the filtrate was concentrated to afford 3.22 g (88%) of product (B) (FIG. 9B). This material was used in the next reaction without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.2 - 1.6 (m, 17H), 2.6 - 2.8 (m, 1H), 3.0 - 3.3 (m, 4H), 3.58 - 3.62 (m, 1H), 4.55 (br s, 1H), 7.2 - 7.4 (m, 5H).

Preparation of (C)

Reagent (C) (FIG. 9C) was prepared by the following procedure. To a solution of concentrated sulfuric acid (75 ml) in distilled water (350 ml) were added D-leucine (FIG. 10A) (50.0 g, 0.381 mol) and potassium bromide (158 g, 1.33 mol), and the solution was cooled to just below 0°C. To the solution was added sodium nitrite (34.8 g, 0.504 mol) as a solution in distilled water (100 ml), dropwise, over a period of 1 hr. After the addition was complete, the mixture was allowed to stir at 0°C for 1 hr. Dichloromethane was added to the solution and the mixture was stirred for several minutes. The layers were separated and the aqueous layer was extracted using dichloromethane. The layers were separated and the combined organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated to afford 45 g (61%) of the crude product. This material was used in the next reaction without further purification: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92-0.96 (m, 6H), 1.7-1.85 (m, 1H), 1.90-1.95 (m, 2H), 4.28 (t, 1H, J=7.6 Hz).

To a cold (-78°C) solution of the bromo-acid (45.6 g, 0.233 mol), produced above, in dichloromethane (200 ml) was introduced isobutene (200 ml) using a cold finger (-78°C). To the solution was added concentrated sulfuric acid (1.5 ml), dropwise, and the solution was allowed to stir and to warm to 25°C over 17 hr. The solution was concentrated to one-half of the original volume by removing the solvent under reduced pressure. The resulting solution was washed using 10% aqueous $NaHCO_3$ (2 x 200 ml) and the layers were separated. The organic layer was dried ($MgSO_4$) and filtered and the filtrate was concentrated under reduced pressure to afford 47 g (81%) of the crude product (FIG. 10B). This material was used in the next reaction without further purification: ^1H NMR (300 MHz, $CDCl_3$) δ 0.85-0.95 (m, 6H), 1.43 (s, 9H), 1.6-1.8 (m, 1H), 1.80-1.85 (m, 2H), 4.13 (t, 1H, J=7.6 Hz).

To a solution of dibenzyl malonate (46.5 g, 0.186 mol) in DMF (80 ml) at 25°C was added potassium *tert*-butoxide (20.7. 0.184 mol), portionwise, and with cooling (occasional icebath). After formation of a homogeneous solution, the solution was cooled (0°C) and the bromo-ester (4.77 g, 0.189 mol) was added dropwise as a solution in DMF (80 ml). After the addition was complete, the solution was allowed to stir at 5°C for 4 days. The mixture was partitioned between ethyl acetate and saturated aqueous ammonium chloride and the layers were separated. The aqueous layer was extracted using ethyl acetate and the layers were separated. The combined organic layer was concentrated under reduced pressure and the residue was dissolved in diethyl ether and was washed using brine. The layers were separated and the organic layer was dried ($MgSO_4$) and filtered and the filtrate was concentrated. The residue was purified using flash column chromatography (gradient elution using 97.5% hexane:ethyl acetate to 90% hexane:ethyl acetate) on 230-400 mesh silica gel to afford 50 g (60%) of the product (FIG. 10C). ^1H NMR (300 MHz, $CDCl_3$) δ 0.80 -0.90 (m, 6H), 1.0-1.1 (m, 1H), 1.40 (s, 9H),

1.45-1.60, (m, 2H), 3.0-3.1 (m, 1H), 3.75, (d, 1H, J=12 Hz), 5.0-5.2 (m, 4H), 7.2-7.4 (m, 10H); MS (positive ion FAB) m/z=455 ($[M+H]^+$).

To the *tert*-butyl ester (34.2 g, 0.0753 mol) was added a solution (95:5) of TFA:H_2O (52.5 ml) and the solution was stored at 0°C for 12 hr. The TFA was removed under reduced pressure and the residue was diluted using dichloromethane. The solution was washed using brine and the layers were separated. The organic layer was dried ($MgSO_4$) and filtered and the filtrate was concentrated to afford 30 g (100%) of the crude product (FIG. 10D, also shown as C in FIG 9). This material was used in the next reaction without further purification: ^1H NMR (300 MHz, $CDCl_3$) δ 0.80-0.85 (m, 6H), 1.1-1.2 (m, 1H), 1.5-1.7 (m, 2H), 3.10-3.25 (m, 1H), 3.8, (d, 1H, J=11 Hz), 5.0-5.2 (m, 4H), 7.2-7.4 (m, 10H), 10.0-10.2 (br s, 1H).

Preparation of (D)

To a solution of the acid (C) (FIG. 9C) (3.10 g, 0.077 mol) in DMF (20 ml) were added HOBt•H_2O (1.36 g, 0.00886 mol), 4-methylmorpholine (0.947 ml, 0.00886 mol), and product (B) (FIG. 9B) (3.22 g, 0.00886 mol) as a solution in THF (20 ml), and the solution was stirred at 25°C for 16 hr. The mixture was filtered and the filtrate was concentrated under reduced pressure to afford an oily residue. The residue was dissolved in EtOAc, washed using aqueous 10% citric acid solution, and the layers separated. The organic layer was washed using an aqueous solution of 10% $NaHCO_3$ and the layers were separated. The organic layer was washed using a saturated aqueous solution of NaCl and the layers were separated. The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated. The residue was purified using flash column chromatography (gradient elution using 100% CH_2Cl_2 to 30% Et_2O:CH_2Cl_2) on 230-400 mesh silica gel to afford 3.70 g (64%) of the major diastereomer (D) (FIG. 9D) and 1.25 g (20%) of the minor diastereomer. ^1H NMR data is reported for the major

diastereomer only. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.74 (d, 3H, J = 2.7 Hz), 0.76 (d, 3H, 2.7 Hz), 1.0 - 1.5 (m, 11H), 1.40 (s, 9H), 2.8 - 3.2 (m, 6H), 3.8 (d, 1H, J = 9.3 Hz), 4.4 - 4.6 (m, 2H), 5.0 - 5.2 (m, 4H), 5.7 (t, 1H, J = 6 Hz), 6.58 (d, 1H, J = 7.5 Hz), 7.2 - 7.4 (m, 15H).

Preparation of (E)

To a solution of (D) (FIG. 9D) (2.89 g, 0.00389 mol) in EtOH (21 ml) was added ammonium formate (1.23 g, 0.0195 mol). To the mixture was added 10% Pd/C (578 mg) as a slurry in isopropanol (5.25 ml), and the mixture was stirred at 25°C for 45 min. The catalyst was removed by filtration, the filtrate was treated with piperidine (0.423 ml, 0.00428 mol), and the solution was stirred at 25°C for 15 min before the addition of aqueous 37% formaldehyde (2.00 ml, 0.0245 mol). After the solution had stirred at 25°C for 19 hr, the solution was warmed to reflux temperature and was stirred for 1 hr. The solution was allowed to cool to 25°C, the solvent was removed under reduced pressure, and the residue was dissolved in EtOAc and was acidified using aqueous 10% citric acid solution. The layers were separated and the organic layer was washed using aqueous 1% K_2CO_3. The layers were separated, and the aqueous layer was acidified to pH 4 using 6N HCl and was extracted using CH_2Cl_2. The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated. The residue was purified using flash column chromatography (elution using 25% MeOH:EtOAc) to afford 1.15 g (56%) of product (E) (FIG 9E). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.73 (d, 3H, J = 6 Hz), 0.78 (d, 3H, J = 6 Hz), 1.0 - 1.6 (m, 11H), 1.4 (s, 9H), 2.6 - 3.0 (m, 6H), 3.3 - 3.4 (m, 1H), 4.35 (q, 1H, J = 6 Hz), 5.10 (s, 1H), 5.80 (s, 1H), 6.78 (t, 1H, J = 5.3 Hz), 7.0 - 7.2 (m, 5H), 7.80 (t, 1H, J = 5.2 Hz), 8.35 (br s, 1H); MS (positive ion FAB) m/z = 538 ([M+Li]$^+$).

Preparation of (F)

To the α,β-unsaturated acid (E) (1.15 g, 0.00216 mol) was added thiophenol (7.32 ml, 0.0714 mol) and the mixture was stirred in the dark at 60°C for 1 day. The solution was allowed to cool to 25°C and Et_2O was added to precipitate the product. The product was collected by filtration, the solid was washed using ether, and was dried under vacuum to afford 0.940 g (65%) of a 3.5:1 mixture of the diastereomeric products (F) (FIG. 9F). This crude mixture of diastereomers was used in the next reaction without further purification. ^1H NMR (300 MHz, DMSO-d_6) δ 0.70 (d, 3H, J = 6 Hz), 0.78 (d, 3H, J = 6 Hz), 1.1 - 1.5 (m, 20H), 2.2 - 3.2 (m, 10H), 4.5 - 4.6 (m, 1H), 6.75 (t, 1H, J = 5.4 Hz), 6.9 - 7.3 (m, 10H), 7.92 (t, 1H, J = 5.3 Hz), 8.40 (d, 1H, J = 8.5 Hz).

Preparation of (G)

To the acid (F) (400 mg, 0.623 mmol) in CH_2Cl_2 (3.0 ml) and DMF (0.76 ml) was added HOBt•H_2O (114 mg, 0.748 mmol) and the mixture was cooled to 0°C, whereupon WSCDI (143 mg, 0.748 mmol) and 4-methylmorpholine (82 ml, 0.748 mmol) were added. The mixture was stirred for 1 hr at 0°C to ensure complete formation of the activated ester. Hydroxylamine hydrochloride (65 mg, 0.934 mmol) and 4-methylmorpholine (103 ml, 0.934 mmol) were added as a solution in DMF (2.0 ml) dropwise, and the mixture was stirred for 1 hr. The solution was poured into a mixture of H_2O (7.5 ml), Et_2O (7.5 ml), and hexane (7.5 ml), and the product precipitated. The precipitate was collected by filtration, and the solid was washed using hexane and was dried under vacuum to afford 204 mg (50%) of the diastereomeric products (G) (FIG. 9G). The diastereomers were separated using RP HPLC to afford 160 mg (39%) of the major diastereomer. Analytical data are reported for the major diastereomer only. ^1H NMR (300 MHz, DMSO-d_6) δ 0.71 (d, 3H, J = 6.3 Hz), 0.78 (d, 3H, J = 6.3 Hz), 1.1 - 1.5 (m, 11H), 1.3 (s, 9H), 2.0 - 3.0 (m, 10H), 4.62 (1H), 6.75 (t,

1H, J = 5.4 Hz), 6.9 - 7.3 (m, 10H), 7.82 (t, 1H, J =, 5.3 Hz), 8.38 (d, 1H, J = 8.8 Hz), 8.92 (br s, 1H), 10.44 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 21.5, 24.1, 25.0, 25.9, 26.0, 28.1, 28.2, 29.0, 29.4, 32.1, 37.5, 45.7, 46.1, 54.0, 77.29, 125.09, 126.29, 127.06, 127.86, 128.77, 129.08, 136.57, 137.94, 155.64, 168.22, 170.87, 172.73; MS (positive ion FAB) m/z 657 ([M+H]$^+$).

Preparation of (H)

To the major diastereomer (G) (160 mg, 0.244 mmol) was added TFA (2.5 ml) and the solution was stirred in a tightly stoppered flask for 20 min. The TFA was removed under reduced pressure and the residue was purified using RP HPLC to afford, after lyophilization, 125 mg (77%) of the TFA salt (H) (FIG. 9H), referred to hereinafter as GI 193463A. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.72 (d, 3H, J = 6.4 Hz), 0.79 (d, 3H, J = 6.4 Hz), 1.1 - 1.6 (m, 10H), 2.0 - 3.2 (m, 10H), 4.6 (m, 1H), 68 - 7.3 (m, 10H), 7.6 (br s, 3H), 7.86 (t, 1H, J = 5.4 Hz), 8.37 (d, 1H, J = 8.8 Hz), 8.83, (s, 1H), 10.44 (s, 1H); MS (positive ion FAB) m/z 557 ([M+H]$^+$).

Preparation of (I)

The biotin derivative of GI 193463A (FIG. 9I), was prepared as follows. GI 193463A (20 mg) was dissolved in 0.6 ml dimethyl formamide and treated with 9 μl triethylamine and 9 mg immunopure NHS-SS biotin (Pierce). After stirring overnight at rm temp, 2 ml water was added and the resulting solid was collected and purified by thin layer chromatography (silica gel) eluted with 10% methanol in dichloromethane to yield 3 mg of final product.

8. EXAMPLE: SUBSTRATE SPECIFICITY OF A PARTIALLY PURIFIED HUMAN TNFα-CON

Experiments were carried out to determine the substrate specificity of a partially purified human TNFα-

con. Partially purified human TNFα-con was generated from microsomal fractions containing convertase activity as follows.

Mono Mac 6 cells (Ziegler-Heitbrook *et al.*, 1988, Int. J. Cancer, 41:456-461) were grown in RPMI 1640 medium containing 10% fetal bovine serum, 0.1% pluronic, penicillin/streptomycin (50 units/ml of each), and L-glutamine (2 mM). Cells (8.2×10^{10}) were sedimented, washed in 50 mM HEPES buffer, pH 7.5, containing 0.25 M sucrose and 2 mM $MgCl_2$, and sedimented. Pellet (260 ml) was resuspended to 600 ml in cold wash buffer containing protease inhibitors (1 mM AEBSF, 10 μM E-64, 10 μM leupeptin, 1 μM pepstatin, 10 μM phosphoramidon, and 50 μM DCI). The suspension was pressurized under 1,000 psi N_2 in a cavitator for 30 min with stirring at 4°C. Pressure was released over 1-2 min with collection of approx. 90% of the lysate. Cell breakage was greater than 80% as determined by trypan blue exclusion.

The broken cell suspension was sedimented in a GS-3 rotor at 3,500 rpm for 10 min. The supernatant (400 ml) was centrifuged in a TFA 20.25 rotor at 20,000 rpm for 45 min at 4°C. The resulting pellet (50 ml) was resuspended to a total volume of 750 ml in 10 mM HEPES buffer, pH 7.5, containing 0.2 M NaCl, 1.2% NP-40, and protease inhibitors as above, except DCI, via dounce homogenization and stirring for 30 min at 4°C. The resuspended material was then centrifuged in a TFA 20.25 rotor at 20,000 rpm for 45 min at 4°C. The resulting pellet had a volume of less than 10 ml.

The supernatant (approx. 750 ml) was passed at 10 ml/min over a column containing 100 ml packed conA-sepharose (Pharmacia) which had been previously equilibrated with 10 mM HEPES buffer, pH 7.5, containing 0.2 M NaCl and 1% NP-40. The loaded column was washed with 200 ml of equilibration buffer, followed by 800 ml of the same buffer but without NaCl. Material was retained overnight at 4°C on the conA column.

Protein was eluted from the conA column by passing 850 ml of 10 mM HEPES buffer, pH 7.5, containing 1% NP-40, 0.25 M methyl-α-D-mannopyranoside, and protease inhibitors as above, except DCI, over the column at 10 ml/min. The eluate was collected and passed over a column containing 2 ml packed POROS™ HQ anion exchanger at 5 ml/min which had been previously equilibrated with 10 mM HEPES buffer, pH 7.5, containing 1% NP-40. The column was washed with 10 ml of equilibration buffer. The buffer level over the packed bed was lowered to just cover the matrix and the inlet tube was flushed with 10 mM HEPES buffer, pH 7.5, containing 0.5 M NaCl, 1 % NP-40, and protease inhibitors as above, except DCI (high salt buffer). Several milliliters of high salt buffer were manually loaded atop the bed column and the inlet was reconnected. High salt buffer was passed over the column at 1 ml/min, and ten 1 ml fractions were collected. Fractions 2 and 3, which had a distinct coloration, were pooled and contained 15.4 mg/ml protein (Micro BCA Kit; BioRad), as well as considerable TNFα-con activity.

To exchange the detergent from NP-40 to dodecylmaltoside (ddm), the enzyme was desalted by dialysis in 10 mM HEPES, pH 7.5, 1% ddm and protease inhibitors. The enzyme was then loaded onto a 200 µl POROS™ HQ column, and eluted off with the above buffer containing 0.5 M NaCl. Fractions containing convertase activity were used in substrate mapping experiments.

Synthetic peptides were prepared that had substitutions at P1′ (Val) or P2′ (Arg) in the following substrate (SEQ ID NO 16):

Biotin-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys-(DNP)-NH2
P4 P3 P2 P1 P1′ P2′ P3′ P4′ P5′

One thousand peptides were assembled in one hundred pools of ten peptides each, via the Fmoc solid phase strategy (Atherton *et al.*, 1989, Solid phase synthesis: a practical approach, IRL Press, Oxford). Assembly was

performed on 5 grams Rink Amide resin (NovaBiochem, lot no. A12697, cat. no. 01-64-0013, 0.46 mmol/gm) by a combination of manual and automated synthesis techniques. Assembly from the C-terminus to P3'(Ser) was performed batchwise in a reaction shaker. The resin was transferred to an Advanced ChemTech 357 MPS for splitting to twenty reaction vessels, coupling of P2', recombining and splitting to two 96-reaction vessel blocks for completion of assembly and subsequent cleavage on an Advanced ChemTech 496 MOS.

An aliquot of substrate was removed and added to an enzyme preparation to a final concentration for each substrate of 1 μM. The fluorescent substrate, NBD-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Lys(DMC)-Ser-Arg-NH_2 (SEQ ID NO 17) (10 μM) was added to follow the reaction by monitoring fluorescence at 370 Nm (excitation), 460 Nm (emission). In addition, cleavage of substrates could be compared to the standard substrate, Biotin-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 16), which was included at 1 μM. The substrates were incubated with enzyme preparation at 37°C for 0.5-4 hr and the reaction quenched by adding an equal volume of 1% HFBA. Samples were passed over a POROS™ avidin column and the products subjected to LC/MS to determine relative intensities of components released in the reaction mixture. The relative reactivities of peptides modified at P1' and P2' are shown in Table 1 below.

Table 1

Substrate Specificity Of A Partially Purified Human TNFα-Con

Substrate	Relative V/K
Biotin-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 18)	1
Biotin-Leu-Ala-Gln-Ala-Phenylgly-Ala-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 19)	1.7
Biotin-Leu-Ala-Gln-Ala-HomoPhe-Ala-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 20)	1.6
Biotin-Leu-Ala-Gln-Ala-Phenylgly-Arg-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 21)	1.5
Biotin-Leu-Ala-Gln-Ala-3-(3-pyridyl)-Ala-His-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 22)	0.5
Biotin-Leu-Ala-Gln-Ala-NorLeu-Arg-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 23)	1.3
Biotin-Leu-Ala-Gln-Ala-NorLeu-Ala-Ser-Ser-Lys-(DNP)-NH_2 (SEQ ID NO 24)	0.7

As can be seen from the data, substrates substituted with natural or unnatural amino acids at P1′ or P2′ are recognized and cleaved by partially purified human TNFα-con.

A second set of experiments were conducted to determine the effect of substrate length on the proteolytic ability of a partially purified human TNFα-con. The DNP-labeled substrates listed in Table 2 below were obtained from Zeneca/Cambridge Research Biochemicals. In individual reactions, substrates at a concentration of 10 μM were reacted with a microsomal enzyme preparation, in RB buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 μM $ZnCl_2$ and 2 mM $CaCl_2$) containing 10 μM leupeptin. Control reactions were run with 10 μM of a hydroxamate inhibitor of TNFα-con (GI 129471X) to insure that products produced in test reactions were generated as a result of TNFα-con activity. Samples were run from 0.5-4 hr at 37°C, and quenched by adding an equal volume of 1% HFBA. Samples were chromatographed on a C18 Vydac column with a 0.1% HFBA water/acetonitrile gradient from 22% to 36% acetonitrile. Product was monitored by measuring absorbance at 380 nM. Relative activities, V/K, for various sized substrates are shown in Table 2 below. The results show that if Ser-Pro-Leu is removed, the peptide cannot serve as a substrate, whereas truncations at the carboxy terminus can take place up to P4′ without a substantial loss in cleavability.

Table 2

Cleavage of Various Sized Substrates
By A Partially Purified Human TNFα-Con

Substrates	Relative V/K
DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH_2 (SEQ ID NO 25)	1
DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-NH_2 (SEQ ID NO 26)	0.6
DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-NH_2 (SEQ ID NO 27)	0.5
DNP-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-NH_2 (SEQ ID NO 28)	0.6
DNP-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH_2 (SEQ ID NO 29)	1.5
DNP-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH2 (SEQ ID NO 30)	1
DNP-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-NH_2 (SEQ ID NO 31)	no turnover

All patents, patent applications, and publications cited above are incorporated herein by reference in their entirety.

The present invention is not to be limited in scope by the specific embodiments described, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent compositions and methods are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the field of molecular biology, medicine or

related fields from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: X, Inventor
Y, Inventor
Z, Inventor

(ii) TITLE OF INVENTION: TUMOR NECROSIS FACTOR ALPHA CONVERTASE

(iii) NUMBER OF SEQUENCES: 39

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Glaxo Wellcome
(B) STREET: Five Moore Drive
(C) CITY: Research Triangle Park
(D) STATE: North Carolina
(E) COUNTRY: U.S.A.
(F) ZIP: 27709

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) TELEX: 802813

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2942 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 164..2635

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGAGCCTGGC GGTAGAATCT TCCCAGTAGG CGGCGCGGGA GGGAAAAGAG GATTGAGGGG 60

CTAGGCCGGG CGGATCCCGT CCTCCCCCGA TGTGAGCAGT TTTCCGAAAC CCCGTCAGGC 120

GAAGGCTGCC CAGAGAGGTG GAGTCGGTAG CGGGGCCGGG AAC ATG AGG CAG TCT 175
Met Arg Gln Ser

1

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CTC CTA TTC CTG ACC AGC GTG GTT CCT TTC GTG CTG GCG CCG CGA CCT          223
Leu Leu Phe Leu Thr Ser Val Val Pro Phe Val Leu Ala Pro Arg Pro
  5                  10                  15                  20

CCG GAT GAC CCG GGC TTC GGC CCC CAC CAG AGA CTC GAG AAG CTT GAT          271
Pro Asp Asp Pro Gly Phe Gly Pro His Gln Arg Leu Glu Lys Leu Asp
              25                  30                  35

TCT TTG CTC TCA GAC TAC GAT ATT CTC TCT TTA TCT AAT ATC CAG CAG          319
Ser Leu Leu Ser Asp Tyr Asp Ile Leu Ser Leu Ser Asn Ile Gln Gln
          40                  45                  50

CAT TCG GTA AGA AAA AGA GAT CTA CAG ACT TCA ACA CAT GTA GAA ACA          367
His Ser Val Arg Lys Arg Asp Leu Gln Thr Ser Thr His Val Glu Thr
      55                  60                  65

CTA CTA ACT TTT TCA GCT TTG AAA AGG CAT TTT AAA TTA TAC CTG ACA          415
Leu Leu Thr Phe Ser Ala Leu Lys Arg His Phe Lys Leu Tyr Leu Thr
  70                  75                  80

TCA AGT ACT GAA CGT TTT TCA CAA AAT TTC AAG GTC GTG GTG GTG GAT          463
Ser Ser Thr Glu Arg Phe Ser Gln Asn Phe Lys Val Val Val Val Asp
 85                  90                  95                 100

GGT AAA AAC GAA AGC GAG TAC ACT GTA AAA TGG CAG GAC TTC TTC ACT          511
Gly Lys Asn Glu Ser Glu Tyr Thr Val Lys Trp Gln Asp Phe Phe Thr
                105                 110                 115

GGA CAC GTG GTT GGT GAG CCT GAC TCT AGG GTT CTA GCC CAC ATA AGA          559
Gly His Val Val Gly Glu Pro Asp Ser Arg Val Leu Ala His Ile Arg
            120                 125                 130

GAT GAT GAT GTT ATA ATC AGA ATC AAC ACA GAT GGG GCC GAA TAT AAC          607
Asp Asp Asp Val Ile Ile Arg Ile Asn Thr Asp Gly Ala Glu Tyr Asn
        135                 140                 145

ATA GAG CCA CTT TGG AGA TTT GTT AAT GAT ACC AAA GAC AAA AGA ATG          655
Ile Glu Pro Leu Trp Arg Phe Val Asn Asp Thr Lys Asp Lys Arg Met
    150                 155                 160

TTA GTT TAT AAA TCT GAA GAT ATC AAG AAT GTT TCA CGT TTG CAG TCT          703
Leu Val Tyr Lys Ser Glu Asp Ile Lys Asn Val Ser Arg Leu Gln Ser
165                 170                 175                 180

CCA AAA GTG TGT GGT TAT TTA AAA GTG GAT AAT GAA GAG TTG CTC CCA          751
Pro Lys Val Cys Gly Tyr Leu Lys Val Asp Asn Glu Glu Leu Leu Pro
                185                 190                 195

AAA GGG TTA GTA GAC AGA GAA CCA CCT GAA GAG CTT GTT CAT CGA GTG          799
Lys Gly Leu Val Asp Arg Glu Pro Pro Glu Glu Leu Val His Arg Val
            200                 205                 210

AAA AGA AGA GCT GAC CCA GAT CCC ATG AAG AAC ACG TGT AAA TTA TTG          847
Lys Arg Arg Ala Asp Pro Asp Pro Met Lys Asn Thr Cys Lys Leu Leu
        215                 220                 225

GTG GTA GCA GAT CAT CGC TTC TAC AGA TAC ATG GGC AGA GGG GAA GAG          895
Val Val Ala Asp His Arg Phe Tyr Arg Tyr Met Gly Arg Gly Glu Glu
    230                 235                 240

AGT ACA ACT ACA AAT TAC TTA ATA GAG CTA ATT GAC AGA GTT GAT GAC          943
Ser Thr Thr Thr Asn Tyr Leu Ile Glu Leu Ile Asp Arg Val Asp Asp
245                 250                 255                 260

ATC TAT CGG AAC ACT TCA TGG GAT AAT GCA GGT TTT AAA GGC TAT GGA          991
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Ile Tyr Arg Asn Thr Ser Trp Asp Asn Ala Gly Phe Lys Gly Tyr Gly
                265                 270                 275

ATA CAG ATA GAG CAG ATT CGC ATT CTC AAG TCT CCA CAA GAG GTA AAA      1039
Ile Gln Ile Glu Gln Ile Arg Ile Leu Lys Ser Pro Gln Glu Val Lys
            280                 285                 290

CCT GGT GAA AAG CAC TAC AAC ATG GCA AAA AGT TAC CCA AAT GAA GAA      1087
Pro Gly Glu Lys His Tyr Asn Met Ala Lys Ser Tyr Pro Asn Glu Glu
        295                 300                 305

AAG GAT GCT TGG GAT GTG AAG ATG TTG CTA GAG CAA TTT AGC TTT GAT      1135
Lys Asp Ala Trp Asp Val Lys Met Leu Leu Glu Gln Phe Ser Phe Asp
    310                 315                 320

ATA GCT GAG GAA GCA TCT AAA GTT TGC TTG GCA CAC CTT TTC ACA TAC      1183
Ile Ala Glu Glu Ala Ser Lys Val Cys Leu Ala His Leu Phe Thr Tyr
325                 330                 335                 340

CAA GAT TTT GAT ATG GGA ACT CTT GGA TTA GCT TAT GTT GGC TCT CCC      1231
Gln Asp Phe Asp Met Gly Thr Leu Gly Leu Ala Tyr Val Gly Ser Pro
                345                 350                 355

AGA GCA AAC AGC CAT GGA GGT GTT TGT CCA AAG GCT TAT TAT AGC CCA      1279
Arg Ala Asn Ser His Gly Gly Val Cys Pro Lys Ala Tyr Tyr Ser Pro
            360                 365                 370

GTT GGG AAG AAA AAT ATC TAT TTG AAT AGT GGT TTG ACG AGC ACA AAG      1327
Val Gly Lys Lys Asn Ile Tyr Leu Asn Ser Gly Leu Thr Ser Thr Lys
        375                 380                 385

AAT TAT GGT AAA ACC ATC CTT ACA AAG GAA GCT GAC CTG GTT ACA ACT      1375
Asn Tyr Gly Lys Thr Ile Leu Thr Lys Glu Ala Asp Leu Val Thr Thr
    390                 395                 400

CAT GAA TTG GGA CAT AAT TTT GGA GCA GAA CAT GAT CCG GAT GGT CTA      1423
His Glu Leu Gly His Asn Phe Gly Ala Glu His Asp Pro Asp Gly Leu
405                 410                 415                 420

GCA GAA TGT GCC CCG AAT GAG GAC CAG GGA GGG AAA TAT GTC ATG TAT      1471
Ala Glu Cys Ala Pro Asn Glu Asp Gln Gly Gly Lys Tyr Val Met Tyr
                425                 430                 435

CCC ATA GCT GTG AGT GGC GAT CAC GAG AAC AAT AAG ATG TTT TCA AAC      1519
Pro Ile Ala Val Ser Gly Asp His Glu Asn Asn Lys Met Phe Ser Asn
            440                 445                 450

TGC AGT AAA CAA TCA ATC TAT AAG ACC ATT GAA AGT AAG GCC CAG GAG      1567
Cys Ser Lys Gln Ser Ile Tyr Lys Thr Ile Glu Ser Lys Ala Gln Glu
        455                 460                 465

TGT TTT CAA GAA CGC AGC AAT AAA GTT TGT GGG AAC TCG AGG GTG GAT      1615
Cys Phe Gln Glu Arg Ser Asn Lys Val Cys Gly Asn Ser Arg Val Asp
    470                 475                 480

GAA GGA GAA GAG TGT GAT CCT GGC ATC ATG TAT CTG AAC AAC GAC ACC      1663
Glu Gly Glu Glu Cys Asp Pro Gly Ile Met Tyr Leu Asn Asn Asp Thr
485                 490                 495                 500

TGC TGC AAC AGC GAC TGC ACG TTG AAG GAA GGT GTC CAG TGC AGT GAC      1711
Cys Cys Asn Ser Asp Cys Thr Leu Lys Glu Gly Val Gln Cys Ser Asp
                505                 510                 515

AGG AAC AGT CCT TGC TGT AAA AAC TGT CAG TTT GAG ACT GCC CAG AAG      1759
Arg Asn Ser Pro Cys Cys Lys Asn Cys Gln Phe Glu Thr Ala Gln Lys
            520                 525                 530
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AAG TGC CAG GAG GCG ATT AAT GCT ACT TGC AAA GGC GTG TCC TAC TGC          1807
Lys Cys Gln Glu Ala Ile Asn Ala Thr Cys Lys Gly Val Ser Tyr Cys
        535                 540                 545

ACA GGT AAT AGC AGT GAG TGC CCG CCT CCA GGA AAT GCT GAA GAT GAC          1855
Thr Gly Asn Ser Ser Glu Cys Pro Pro Pro Gly Asn Ala Glu Asp Asp
    550                 555                 560

ACT GTT TGC TTG GAT CTT GGC AAG TGT AAG GAT GGG AAA TGC ATC CCT          1903
Thr Val Cys Leu Asp Leu Gly Lys Cys Lys Asp Gly Lys Cys Ile Pro
565                 570                 575                 580

TTC TGC GAG AGG GAA CAG CAG CTG GAG TCC TGT GCA TGT AAT GAA ACT          1951
Phe Cys Glu Arg Glu Gln Gln Leu Glu Ser Cys Ala Cys Asn Glu Thr
                585                 590                 595

GAC AAC TCC TGC AAG GTG TGC TGC AGG GAC CTT TCT GGC CGC TGT GTG          1999
Asp Asn Ser Cys Lys Val Cys Cys Arg Asp Leu Ser Gly Arg Cys Val
            600                 605                 610

CCC TAT GTC GAT GCT GAA CAA AAG AAC TTA TTT TTG AGG AAA GGA AAG          2047
Pro Tyr Val Asp Ala Glu Gln Lys Asn Leu Phe Leu Arg Lys Gly Lys
        615                 620                 625

CCC TGT ACA GTA GGA TTT TGT GAC ATG AAT GGC AAA TGT GAG AAA CGA          2095
Pro Cys Thr Val Gly Phe Cys Asp Met Asn Gly Lys Cys Glu Lys Arg
    630                 635                 640

GTA CAG GAT GTA ATT GAA CGA TTT TGG GAT TTC ATT GAC CAG CTG AGC          2143
Val Gln Asp Val Ile Glu Arg Phe Trp Asp Phe Ile Asp Gln Leu Ser
645                 650                 655                 660

ATC AAT ACT TTT GGA AAG TTT TTA GCA GAC AAC ATC GTT GGG TCT GTC          2191
Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val
                665                 670                 675

CTG GTT TTC TCC TTG ATA TTT TGG ATT CCT TTC AGC ATT CTT GTC CAT          2239
Leu Val Phe Ser Leu Ile Phe Trp Ile Pro Phe Ser Ile Leu Val His
            680                 685                 690

TGT GTG GAT AAG AAA TTG GAT AAA CAG TAT GAA TCT CTG TCT CTG TTT          2287
Cys Val Asp Lys Lys Leu Asp Lys Gln Tyr Glu Ser Leu Ser Leu Phe
        695                 700                 705

CAC CCC AGT AAC GTC GAA ATG CTG AGC AGC ATG GAT TCT GCA TCG GTT          2335
His Pro Ser Asn Val Glu Met Leu Ser Ser Met Asp Ser Ala Ser Val
    710                 715                 720

CGC ATT ATC AAA CCC TTT CCT GCG CCC CAG ACT CCA GGC CGC CTG CAG          2383
Arg Ile Ile Lys Pro Phe Pro Ala Pro Gln Thr Pro Gly Arg Leu Gln
725                 730                 735                 740

CCT GCC CCT GTG ATC CCT TCG GCG CCA GCA GCT CCA AAA CTG GAC CAC          2431
Pro Ala Pro Val Ile Pro Ser Ala Pro Ala Ala Pro Lys Leu Asp His
                745                 750                 755

CAG AGA ATG GAC ACC ATC CAG GAA GAC CCC AGC ACA GAC TCA CAT ATG          2479
Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Met
            760                 765                 770

GAC GAG GAT GGG TTT GAG AAG GAC CCC TTC CCA AAT AGC AGC ACA GCT          2527
Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Thr Ala
        775                 780                 785

GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACC AGA AGT GAA          2575
Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu
    790                 795                 800
```

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AAG GCT GCC TCC TTT AAA CTG CAG CGT CAG AAT CGT GTT GAC AGC AAA      2623
Lys Ala Ala Ser Phe Lys Leu Gln Arg Gln Asn Arg Val Asp Ser Lys
805                 810                 815                 820

GAA ACA GAG TGC TAATTTAGTT CTCAGCTCTT CTGACTTAAG TGTGCAAAAT        2675
Glu Thr Glu Cys

ATTTTTATAG ATTTGACCTA CAATCAATCA CAGCTTATAT TTTGTGAAGA CTGGGAAGTG  2735

ACTTAGCAGA TGCTGGTCAT GTGTTTGGAA CTTCCTGCAG GTAAACAGTT CTTGTGTGGG  2795

TTTGGGCCCN TCTCCTTTTG GAAAAGGTAA GGGTGAAGGT GAATCTTGCT TATTNTGGGG  2855

GTTTCAGGTT TNAGTTTTTA AAATATCTTT TGGACCTGTG GGTGNAAAAG CAGAAATACA  2915

GNTGGATTGG GTTATGAGTA TTTACGT                                       2942
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 824 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Arg Gln Ser Leu Leu Phe Leu Thr Ser Val Val Pro Phe Val Leu
  1               5                  10                  15

Ala Pro Arg Pro Pro Asp Asp Pro Gly Phe Gly Pro His Gln Arg Leu
            20                  25                  30

Glu Lys Leu Asp Ser Leu Leu Ser Asp Tyr Asp Ile Leu Ser Leu Ser
        35                  40                  45

Asn Ile Gln Gln His Ser Val Arg Lys Arg Asp Leu Gln Thr Ser Thr
    50                  55                  60

His Val Glu Thr Leu Leu Thr Phe Ser Ala Leu Lys Arg His Phe Lys
65                  70                  75                  80

Leu Tyr Leu Thr Ser Ser Thr Glu Arg Phe Ser Gln Asn Phe Lys Val
                85                  90                  95

Val Val Val Asp Gly Lys Asn Glu Ser Glu Tyr Thr Val Lys Trp Gln
            100                 105                 110

Asp Phe Phe Thr Gly His Val Val Gly Glu Pro Asp Ser Arg Val Leu
        115                 120                 125

Ala His Ile Arg Asp Asp Asp Val Ile Ile Arg Ile Asn Thr Asp Gly
    130                 135                 140

Ala Glu Tyr Asn Ile Glu Pro Leu Trp Arg Phe Val Asn Asp Thr Lys
145                 150                 155                 160

Asp Lys Arg Met Leu Val Tyr Lys Ser Glu Asp Ile Lys Asn Val Ser
                165                 170                 175

Arg Leu Gln Ser Pro Lys Val Cys Gly Tyr Leu Lys Val Asp Asn Glu
            180                 185                 190

Glu Leu Leu Pro Lys Gly Leu Val Asp Arg Glu Pro Pro Glu Glu Leu
```

195 200 205

Val His Arg Val Lys Arg Arg Ala Asp Pro Asp Pro Met Lys Asn Thr
210 215 220

Cys Lys Leu Leu Val Val Ala Asp His Arg Phe Tyr Arg Tyr Met Gly
225 230 235 240

Arg Gly Glu Glu Ser Thr Thr Thr Asn Tyr Leu Ile Glu Leu Ile Asp
245 250 255

Arg Val Asp Asp Ile Tyr Arg Asn Thr Ser Trp Asp Asn Ala Gly Phe
260 265 270

Lys Gly Tyr Gly Ile Gln Ile Glu Gln Ile Arg Ile Leu Lys Ser Pro
275 280 285

Gln Glu Val Lys Pro Gly Glu Lys His Tyr Asn Met Ala Lys Ser Tyr
290 295 300

Pro Asn Glu Glu Lys Asp Ala Trp Asp Val Lys Met Leu Leu Glu Gln
305 310 315 320

Phe Ser Phe Asp Ile Ala Glu Glu Ala Ser Lys Val Cys Leu Ala His
325 330 335

Leu Phe Thr Tyr Gln Asp Phe Asp Met Gly Thr Leu Gly Leu Ala Tyr
340 345 350

Val Gly Ser Pro Arg Ala Asn Ser His Gly Gly Val Cys Pro Lys Ala
355 360 365

Tyr Tyr Ser Pro Val Gly Lys Lys Asn Ile Tyr Leu Asn Ser Gly Leu
370 375 380

Thr Ser Thr Lys Asn Tyr Gly Lys Thr Ile Leu Thr Lys Glu Ala Asp
385 390 395 400

Leu Val Thr Thr His Glu Leu Gly His Asn Phe Gly Ala Glu His Asp
405 410 415

Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln Gly Gly Lys
420 425 430

Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu Asn Asn Lys
435 440 445

Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr Ile Glu Ser
450 455 460

Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val Cys Gly Asn
465 470 475 480

Ser Arg Val Asp Glu Gly Glu Glu Cys Asp Pro Gly Ile Met Tyr Leu
485 490 495

Asn Asn Asp Thr Cys Cys Asn Ser Asp Cys Thr Leu Lys Glu Gly Val
500 505 510

Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys Gln Phe Glu
515 520 525

Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr Cys Lys Gly
530 535 540

Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro Pro Gly Asn
545 550 555 560

```
Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Lys Cys Lys Asp Gly
                  565                 570                 575

Lys Cys Ile Pro Phe Cys Glu Arg Glu Gln Gln Leu Glu Ser Cys Ala
              580                 585                 590

Cys Asn Glu Thr Asp Asn Ser Cys Lys Val Cys Cys Arg Asp Leu Ser
          595                 600                 605

Gly Arg Cys Val Pro Tyr Val Asp Ala Glu Gln Lys Asn Leu Phe Leu
      610                 615                 620

Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met Asn Gly Lys
625                 630                 635                 640

Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp Asp Phe Ile
                  645                 650                 655

Asp Gln Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile
              660                 665                 670

Val Gly Ser Val Leu Val Phe Ser Leu Ile Phe Trp Ile Pro Phe Ser
          675                 680                 685

Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln Tyr Glu Ser
      690                 695                 700

Leu Ser Leu Phe His Pro Ser Asn Val Glu Met Leu Ser Ser Met Asp
705                 710                 715                 720

Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro Gln Thr Pro
                  725                 730                 735

Gly Arg Leu Gln Pro Ala Pro Val Ile Pro Ser Ala Pro Ala Ala Pro
              740                 745                 750

Lys Leu Asp His Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr
          755                 760                 765

Asp Ser His Met Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn
      770                 775                 780

Ser Ser Thr Ala Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val
785                 790                 795                 800

Thr Arg Ser Glu Lys Ala Ala Ser Phe Lys Leu Gln Arg Gln Asn Arg
                  805                 810                 815

Val Asp Ser Lys Glu Thr Glu Cys
              820
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 3
(D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTNCARGAYG TRATHGA 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 3
(D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTNCARGAYG TYATHGA 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 3
(D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCNACDATRT TRTCYGC 17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCNACDATRT TRTCRGC 17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp Lys Leu Ser
1               5                  10                  15

Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val
            20                  25                  30

Leu Val Phe Ser Leu Met Leu Trp Ile
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
GTGCAGGACG TCATCGAGCG GTTCTGGGAG TTCATTGACA AGCTGAGCAT CAATACTTTC      60

GGGAAGTTCC TGGCAGACAA CATCGTCGG                                        89
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2464 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1308

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GAA GCT GAC CTT GTG ACA ACT CAT GAA TTG GGG CAC AAT TTT GGA GCA        48
Glu Ala Asp Leu Val Thr Thr His Glu Leu Gly His Asn Phe Gly Ala
  1               5                  10                  15

GAA CAC GAT CCA GAT GGT TTA GCA GAA TGT GCC CCA AAC GAG GAC CAG        96
Glu His Asp Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln
            20                  25                  30

GGA GGA AAA TAC GTC ATG TAT CCC ATA GCC GTG AGT GGT GAT CAT GAG       144
Gly Gly Lys Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu
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                35                  40                  45

AAC AAC AAG ATG TTT TCA AAC TGC AGT AAA CAG TCC ATC TAT AAG ACC          192
Asn Asn Lys Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr
     50                  55                  60

ATT GAA AGT AAG GCC CAG GAG TGT TTT CAA GAG CGC AGC AAC AAA GTG          240
Ile Glu Ser Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val
 65                  70                  75                  80

TGT GGC AAC TCC AGG GTG GAT GAG GGG GAG GAG TGC GAC CCC GGC ATC          288
Cys Gly Asn Ser Arg Val Asp Glu Gly Glu Glu Cys Asp Pro Gly Ile
                 85                  90                  95

ATG TAC CTG AAC AAC GAC ACC TGC TGC AAC AGC GAC TGC ACC CTG AGG          336
Met Tyr Leu Asn Asn Asp Thr Cys Cys Asn Ser Asp Cys Thr Leu Arg
            100                 105                 110

CCG GGC GTC CAG TGC AGT GAT AGG AAC AGT CCT TGC TGT AAA AAC TGT          384
Pro Gly Val Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys
        115                 120                 125

CAG TTC GAG ACG GCC CAG AAG AAG TGC CAG GAG GCT ATT AAT GCC ACT          432
Gln Phe Glu Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr
    130                 135                 140

TGC AAA GGC GTG TCT TAC TGC ACA GGT AAC AGC AGT GAG TGC CCC CCT          480
Cys Lys Gly Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro
145                 150                 155                 160

CCG GGA AAC GCC GAG GAC GAC ACG GTG TGC CTG GAC CTG GGC AGG TGC          528
Pro Gly Asn Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Arg Cys
                165                 170                 175

AAG GAC GGC AAG TGC GTG CCC TTC TGC GAG CGG GAG CAG CGG CTG GAG          576
Lys Asp Gly Lys Cys Val Pro Phe Cys Glu Arg Glu Gln Arg Leu Glu
            180                 185                 190

TCC TGC GCG TGT AAC GAA ACC GAC CAC TCG TGC AAG GTG TGC TGC CGG          624
Ser Cys Ala Cys Asn Glu Thr Asp His Ser Cys Lys Val Cys Cys Arg
        195                 200                 205

GCC CCC TCG GGC CGT TGG CTG CCC TAC GTG GAC GCC GAA CAG AAG AAC          672
Ala Pro Ser Gly Arg Trp Leu Pro Tyr Val Asp Ala Glu Gln Lys Asn
    210                 215                 220

TTG TTT TTG AGG AAG GGG AAG CCC TGT ACA GTA GGA TTT TGT GAC ATG          720
Leu Phe Leu Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met
225                 230                 235                 240

AAT GGC AAG TGT GAG AAG CGA GTG CAG GAC GTC ATC GAG CGG TTC TGG          768
Asn Gly Lys Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp
                245                 250                 255

GAG TTC ATT GAC AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG GCA          816
Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala
            260                 265                 270

GAC AAC ATC GTG GGC TCC GTC CTG GTG TTC TCC CTG ATG CTC TGG ATC          864
Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile
        275                 280                 285

CCC GTC AGC ATC CTC GTC CAC TGC GTG GAT AAG AAG CTG GAT AAG CAG          912
Pro Val Ser Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln
    290                 295                 300

TAC GAA TCC CTG TCT CTG CTG CAC CCC AGC AAC GTG GAG ATG CTA AGC          960
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```
Tyr Glu Ser Leu Ser Leu Leu His Pro Ser Asn Val Glu Met Leu Ser
305                 310                 315                 320

AGC ATG GAT TCA GCA TCC GTT CGC ATC ATC AAG CCC TTT CCT GCG CCC      1008
Ser Met Asp Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro
                325                 330                 335

CAG ACC CCA GGC CGC CTG CAG CCC CTG CAG CCC CTG CAG CCC GGC CCC      1056
Gln Thr Pro Gly Arg Leu Gln Pro Leu Gln Pro Leu Gln Pro Gly Pro
            340                 345                 350

GTG CTG CCC TCT GCG CCT TCG GTG CCC GTG GCT CCA AAA CTG GAC CAC      1104
Val Leu Pro Ser Ala Pro Ser Val Pro Val Ala Pro Lys Leu Asp His
        355                 360                 365

CAG CGG ATG GAC ACC ATC CAG GAG GAC CCC AGC ACG GAC TCG CAC GTG      1152
Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Val
    370                 375                 380

GAC GAG GAC GGC TTC GAG AAG GAC CCT TTC CCC AAC AGC AGT GCC GCT      1200
Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Ala Ala
385                 390                 395                 400

GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACG AGA AGT GAA      1248
Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu
                405                 410                 415

AAG GCC TCG TCC TTT AAG CTG CAG CGC CAG AGT CGC GTT GAC AGC AAG      1296
Lys Ala Ser Ser Phe Lys Leu Gln Arg Gln Ser Arg Val Asp Ser Lys
            420                 425                 430

GAA ACG GAG TGC TAGTTCAGGG CTGCGTCCGG AGCTCTCGGA CTTGGCGTGC          1348
Glu Thr Glu Cys
        435

AGGATATTTT TATAGATTTG ACCTCAGATC ACTGCGGAGA TCTTGTGAAG ATTTGGGAGA    1408

AAAAAGGAAG TGACTTCCCA GCAGATGCTG GTCATGAGTT TGGACTTCCT CCACCTCCGT    1468

GAGACTGTGT GGGTAGCCCT TTTCCTTTGA AGAGGTAAGG CGAACCTAGC TTACTTTGAG    1528

GCCTTCAGGT TTTAGTCTTT TGATCTTTAA AATATCTTTC AACCTGTGGT GCAGAAGCAG    1588

AAACCACAGC TGGATTATGT TATGACTATT TACGTTTTTG TAAATTACCT TTATATTGAG    1648

AACAGCACTG ATTGTGGAGG TGATCATGTT TTTAAGACAC TGTAACGATC CAATGAACAC    1708

AAAGAGGCAT TTCATCAGTT TCCTGCGAGG ATAACTGGAA CACAGAATAG ATGGATTTCT    1768

TCCCTTCAGG TCAAATAAAG TCCGAGGTGC GTAATGTCAC CCAAATCTGC TTTCTGATGC    1828

AGAACCCGGT TCTACGTTCC CCCCAAAGGT GGGTTCAGGT TCGAGGTATT GCCTAGAACT    1888

TAGAGCAGAA GGGAGACCAC ACATCTGAAT TTAGGAATAA ACTGGGTTAA TGTTTCTTCC    1948

TGTTTCTAAA TGTTCAGAAT TTCTAATTTC TAGAATTAAA ACAATCTAGC TTTATAGTAA    2008

AGATTATAAA TTATAGCCAC ATGAATTGAC AGAATCCTGC TGATTAACTG TTTAGGGCCA    2068

TACCTAGCTG GGCAGTTTTT CTGGGTTTTG TAAGTATCTT CCTGTCTCTC TACGGGTGCC    2128

ACGCTCCTAA AAAACCCACC ATCCACAAAA CCTCAACCCT TCAGAACAGG AGGACGGTCG    2188

CCTCTTCGCA AACACTAGTG GCCCAGACGG TGCGTGCGGT TGGGCGCGCT TGCCAGGCGA    2248

ACAGGGTGCG AGGCGCTGGC CGGGGGTCTG TCCTCAGACC ATTGGTCCCT AGACCCTAGA    2308
```

CCTTCTGGCT CGCGTGCTTC GGCACCGTCT GCGCGCCCTG CGCGAGGGAT AAAAACCATC 2368

CGGCTGAGTT CTGGTTTCAA AAGCTTTTTC CCCTGCTAAG GACTGCAGTG TGCTCACCAG 2428

CCAGCACCCC TTAACAGGTA ACTTGTAAGC TTCGAG 2464

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 436 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Ala Asp Leu Val Thr Thr His Glu Leu Gly His Asn Phe Gly Ala
1 5 10 15

Glu His Asp Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln
20 25 30

Gly Gly Lys Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu
35 40 45

Asn Asn Lys Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr
50 55 60

Ile Glu Ser Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val
65 70 75 80

Cys Gly Asn Ser Arg Val Asp Glu Gly Glu Glu Cys Asp Pro Gly Ile
85 90 95

Met Tyr Leu Asn Asn Asp Thr Cys Cys Asn Ser Asp Cys Thr Leu Arg
100 105 110

Pro Gly Val Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys
115 120 125

Gln Phe Glu Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr
130 135 140

Cys Lys Gly Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro
145 150 155 160

Pro Gly Asn Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Arg Cys
165 170 175

Lys Asp Gly Lys Cys Val Pro Phe Cys Glu Arg Glu Gln Arg Leu Glu
180 185 190

Ser Cys Ala Cys Asn Glu Thr Asp His Ser Cys Lys Val Cys Cys Arg
195 200 205

Ala Pro Ser Gly Arg Trp Leu Pro Tyr Val Asp Ala Glu Gln Lys Asn
210 215 220

Leu Phe Leu Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met
225 230 235 240

Asn Gly Lys Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp
245 250 255

Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala

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            260                 265                 270

Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile
        275                 280                 285

Pro Val Ser Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln
    290                 295                 300

Tyr Glu Ser Leu Ser Leu Leu His Pro Ser Asn Val Glu Met Leu Ser
305                 310                 315                 320

Ser Met Asp Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro
                325                 330                 335

Gln Thr Pro Gly Arg Leu Gln Pro Leu Gln Pro Leu Gln Pro Gly Pro
            340                 345                 350

Val Leu Pro Ser Ala Pro Ser Val Pro Val Ala Pro Lys Leu Asp His
        355                 360                 365

Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Val
    370                 375                 380

Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Ala Ala
385                 390                 395                 400

Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu
                405                 410                 415

Lys Ala Ser Ser Phe Lys Leu Gln Arg Gln Ser Arg Val Asp Ser Lys
            420                 425                 430

Glu Thr Glu Cys
        435
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Val Gln Asp Val Ile Glu
1               5
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Asp Asn Ile Val Gly
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCGGTTCTGG GAGTTCATTG ACAAGCTGAG CATCAATACT TTCGGGAAGT TCCTG 55

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /product= "OTHER"
/note= "X = Dinitrophenylalanine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Xaa Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Val Gln Asp Val Ile Glu Arg Phe Trp Glu Phe Ile Asp Lys Leu Ser
1 5 10 15

Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly
20 25 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Leu Ala Gln Ala Val Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A fluorophore such as NBD is N-terminally linked to serine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 10
(D) OTHER INFORMATION: /note= "DMC (dimethyl coumarin) is linked as a side group to Lys"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Pro Leu Ala Gln Ala Val Arg Ser Lys Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Ala Gln Ala Val Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "X = phenylglycine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Ala Gln Ala Xaa Ala Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "X = Homophenylalanine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Ala Gln Ala Xaa Ala Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "X = Phenylglycine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally bound to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Ala Gln Ala Xaa Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /note= "X = 3-(3-pyridyl) Alanine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally linked to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Ala Gln Ala Xaa His Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /product= "X = Nle"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally bound to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Leu Ala Gln Ala Xaa Arg Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "A biotin molecule is N-terminally linked to leucine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /product= "X = Nle"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine-NH2 is C-terminally bound to lysine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Leu Ala Gln Ala Xaa Ala Ser Ser Lys
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is N-terminally bound to serine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 12
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound to arginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is N-terminally bound to serine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 11
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound

to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ser Pro Leu Ala Gln Ala Val Arg Ser Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dintrophenylalanine is N-terminally bound to serine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 10
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ser Pro Leu Ala Gln Ala Val Arg Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound to serine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is N-terminally bound to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ser Pro Leu Ala Gln Ala Val Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is N-terminally bound to proline"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 11
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound to arginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Pro Leu Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 10
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound to arginine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is N-terminally bound to serine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Leu Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Dinitrophenylalanine is N-terminally linked to alanine"

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "NH2 is C-terminally bound to arginine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Gln Ala Val Arg Ser Ser Ser Arg
1 5

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCTAGAGTCA GGCTCACCAA CC 22

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CACGCGTCGA CTAGTTACCA TCCACCACCA CGACCTTGAA ATTTTGTG 48

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 36
(D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 37
(D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 41
(D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 42
(D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 46
(D) OTHER INFORMATION: /mod_base= i

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION: 47
(D) OTHER INFORMATION: /mod_base= i

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CUACUACUAC UAGGCCACGC GTCGACTAGT ACGGGNNGGG NNGGGNNG 48

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CGGGATCCAT GAGGCAGTCT CTCCTATTCC TGACC 35

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CAGGAAGTTG CGGCCGCTGA CCAGCATCTG CTAAGTCACT TCCCAGTCTT CAC 53

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCGCGCGCGC CATATGTTAG TTTATAAATC TGAAGATATC AAGAATGTTT CACG 54

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CGCGCGCGCG GGATCCCTAT CGTTCAATTA CATCCTGTAC TCGTTTCTCA C 51

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 518 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AAACTGGCTG AACCGGCCTT TTGGTAACGC CACCTTCACT TCTGGGGGCG TCGAACCTGG 60

CGGTAGAATC TTCCCAGTAG GCGGCGCGGG AGGGAAAAGA GGATTGAGGG GCTAGGCCGG 120

GCGGATCCCG TCCTCCCCCG ATGTGAGCAG TTTTCCGAAA CCCCGTCAGG CGAAGGCTGC 180

CCAGAGAGGT GGAGTCGGTA GCGGGGCCGG GAACATGAGG CAGTCTCTCC TATTCCTGAC 240

CAGCGTGGTT CCTTTCGTGC TGGCGCCGCG ACCTCCGGAT GACCCGGGCT TCGGCCCCCA 300

CCAGAGACTC GAGGAGCTTG ATTCTTTGCT CTCAGACTAC GATATTCTCT CTTTATCTAA 360

TATCCAGCAG CATTCGGTAA GAAAAAGAGA TCTACAGACT TCAACACATG TAGAAACACT 420

ACTAACTTTT TCAGCTTTGA AAAGGCATTT TAAATTATAC CTGACATCAA GTACTGAACG 480

TTTTTCACAA AATTTCAAGG TCGTGGTGGT GGATGGTA 518

WHAT IS CLAIMED IS:

1. An isolated DNA sequence encoding a biologically active TNFα-convertase.

2. The isolated DNA sequence of claim 1, wherein the TNFα-convertase is from a mammal.

3. The isolated DNA sequence of claim 2, wherein the TNFα-convertase is from a human.

4. The isolated DNA of claim 3, having the nucleotide sequence shown in FIGURE 1 (SEQ ID NO 1).

5. An isolated DNA, which is capable of hybridizing under highly stringent or moderately stringent conditions to a DNA sequence that is complementary to the DNA sequence of claim 1 (SEQ ID NO 1).

6. A recombinant DNA expression vector comprising the DNA of claim 1.

7. The recombinant DNA expression vector of claim 6, in which the DNA is operatively associated with a regulatory sequence that controls expression of the DNA in a host.

8. The recombinant DNA expression vector of claim 7, which further comprises a DNA sequence encoding a selectable marker or reporter gene product.

9. A host cell containing the recombinant DNA expression vector of claim 6.

10. The host cell of claim 9 that expresses a biologically active TNFα-convertase.

11. A substantially pure mammalian TNFα-convertase.

12. The substantially pure TNFα-convertase of claim 11, wherein the mammal is a human.

13. The substantially pure TNFα-convertase of claim 11, wherein the mammal is a pig.

14. A substantially pure TNFα-convertase having the amino acid sequence shown in FIGURE 1 (SEQ ID NO 2).

15. A fusion protein comprising a TNFα-convertase linked to a heterologous protein or peptide sequence.

16. A method for isolating a recombinant TNFα-convertase, comprising culturing the host cell of claim 9 under conditions conducive to the production of a biologically active TNFα-convertase, and recovering TNFα-convertase from the cell culture.

17. A recombinant TNFα-convertase produced by the method of claim 16.

18. A method for isolating a compound capable of binding to TNFα-convertase, comprising:

(a) immobilizing TNFα-convertase or a portion thereof by conjugation to a solid phase matrix;

(b) contacting the TNFα-convertase-solid phase matrix conjugate of (a) with a material comprising a compound under conditions that permit the compound to bind to the immobilized TNFα-convertase;

(c) removing unbound material from the solid phase matrix;

(d) detecting the presence of compound bound to the TNFα-convertase;

(e) eluting the bound compound from the TNFα-convertase; and

(f) collecting the eluted compound.

19. A compound capable of binding to TNFα-convertase isolated by the method of claim 18.

20. The compound of claim 19, which inhibits a biological activity of TNFα-convertase.

21. A method for producing TNFα-convertase comprising:

(a) transfecting cells with a recombinant DNA expression vector comprising the DNA sequence of claim 1 (SEQ ID NO 1);

(b) growing the cells of step (a) in culture medium under conditions conducive to expression of the DNA sequence and production of TNFα-convertase; and

(c) isolating the TNFα-convertase.

22. The method of claim 21, wherein the TNFα-convertase is isolated by:

(a) contacting the TNFα-convertase with a TNFα-convertase inhibitor that further comprises a biotin moiety under conditions conducive to binding of the TNFα-convertase to the inhibitor-biotin moiety so as to form a TNFα-convertase-inhibitor-biotin conjugate;

(b) contacting the TNFα-convertase-inhibitor-biotin conjugate of (a) with streptavidin bound to a solid phase matrix under conditions conducive to binding of the TNFα-convertase-inhibitor-biotin conjugate to the streptavidin;

(c) removing unbound material; and

(d) eluting the TNFα-convertase-inhibitor-biotin conjugate from the streptavidin or eluting the TNFα-convertase from the inhibitor-biotin conjugate.

23. A method of screening for compounds that modulate the level of TNFα in a mammalian subject, comprising testing compounds for their ability to modulate a biological activity of TNFα-convertase.

24. The method of claim 23, wherein the biological activity of TNFα-convertase is selected from the group consisting of detectable binding to TNFα precursor, or to TNFα, or to a synthetic substrate, conversion of TNFα precursor to mature TNFα, and cleavage of a synthetic substrate.

25. A method of treating a disease or condition characterized by an elevated level of TNFα in the serum or tissues of a mammalian subject, comprising administering an effective amount of a compound that modulates the biological activity of TNFα-convertase to a mammalian subject in need of said treatment.

26. The method of claim 25, wherein the disease or condition is selected from the group consisting of systemic inflammatory response syndrome, reperfusion injury, cardiovascular disease, infectious disease, obstetrical disorders, gynecological disorders, inflammatory disease, autoimmunity, allergic disease, atopic disease, malignancy, transplant complication

27. The method of claim 25, wherein the disease or conditions is selected from the group consisting of septic shock, cachexia, AIDS, graft-versus-host disease, cerebral malaria, Crohn's disease, diabetes, inflammatory

bowel disease, osteoporosis, restenosis, psoriasis, infarction, and rheumatoid arthritis, macular degeneration, osteoarthritis, and multiple sclerosis.

28. The method of claim 25, wherein the disease or condition is infarction due to an ischemic event.

29. A pharmaceutical composition, comprising a compound that inhibits TNFα-convertase and a pharmaceutically acceptable carrier.

30. An oligonucleotide which encodes an antisense sequence complementary to a portion of a TNFα-convertase coding sequence, and which inhibits transcription or translation of the TNFα-convertase coding sequence in a cell.

31. A complex comprising TNFα-convertase having the amino acid sequence shown in Figure 1 (SEQ ID NO 1) or a portion thereof and a therapeutic agent capable of modulating the activity of the TNFα-convertase.

32. An inhibitor of TNFα-convertase, having the chemical structure shown in Figure 8.

33. The inhibitor of claim 32, wherein R comprises a biotin moiety.

34. The inhibitor of claim 33, wherein R is selected from the group consisting of $-(CH_2)_n$-Biotin, where n = 0-10; $-(CH_2)_n$-Imino Biotin, where n = 0-10; $-(CH_2)_n$-S-S-$(CH_2)_n$-Biotin, where n = 0-10; and $-(CH_2)_n$-S-S-(CH_2)n-Imino Biotin, where n = 0-10.

35. A method of isolating TNFα-convertase comprising contacting a preparation comprising TNFα-convertase with the inhibitor of claim 32 under conditions

conducive to the binding of the TNFα-convertase to the inhibitor to form a TNFα-convertase-complex and isolating the TNFα-convertase-complex.

```
CGAGCCTGGC GGTAGAATCT TCCCAGTAGG CGGCGCGGGA GGGAAAAGAG GATTGAGGGG      60

CTAGGCCGGG CGGATCCCGT CCTCCCCCGA TGTGAGCAGT TTTCCGAAAC CCCGTCAGGC     120

GAAGGCTGCC CAGAGAGGTG GAGTCGGTAG CGGGGCCGGG AAC ATG AGG CAG TCT       175
                                                Met Arg Gln Ser
                                                  1

CTC CTA TTC CTG ACC AGC GTG GTT CCT TTC GTG CTG GCG CCG CGA CCT       223
Leu Leu Phe Leu Thr Ser Val Val Pro Phe Val Leu Ala Pro Arg Pro
  5                  10                  15                  20

CCG GAT GAC CCG GGC TTC GGC CCC CAC CAG AGA CTC GAG AAG CTT GAT       271
Pro Asp Asp Pro Gly Phe Gly Pro His Gln Arg Leu Glu Lys Leu Asp
                  25                  30                  35

TCT TTG CTC TCA GAC TAC GAT ATT CTC TCT TTA TCT AAT ATC CAG CAG       319
Ser Leu Leu Ser Asp Tyr Asp Ile Leu Ser Leu Ser Asn Ile Gln Gln
              40                  45                  50

CAT TCG GTA AGA AAA AGA GAT CTA CAG ACT TCA ACA CAT GTA GAA ACA       367
His Ser Val Arg Lys Arg Asp Leu Gln Thr Ser Thr His Val Glu Thr
          55                  60                  65

CTA CTA ACT TTT TCA GCT TTG AAA AGG CAT TTT AAA TTA TAC CTG ACA       415
Leu Leu Thr Phe Ser Ala Leu Lys Arg His Phe Lys Leu Tyr Leu Thr
      70                  75                  80

TCA AGT ACT GAA CGT TTT TCA CAA AAT TTC AAG GTC GTG GTG GTG GAT       463
Ser Ser Thr Glu Arg Phe Ser Gln Asn Phe Lys Val Val Val Val Asp
  85                  90                  95                  100
```

FIG. 1(I)

```
GGT AAA AAC GAA AGC GAG TAC ACT GTA AAA TGG CAG GAC TTC TTC ACT          511
Gly Lys Asn Glu Ser Glu Tyr Thr Val Lys Trp Gln Asp Phe Phe Thr
                105                 110                 115

GGA CAC GTG GTT GGT GAG CCT GAC TCT AGG GTT CTA GCC CAC ATA AGA          559
Gly His Val Val Gly Glu Pro Asp Ser Arg Val Leu Ala His Ile Arg
            120                 125                 130

GAT GAT GAT GTT ATA ATC AGA ATC AAC ACA GAT GGG GCC GAA TAT AAC          607
Asp Asp Asp Val Ile Ile Arg Ile Asn Thr Asp Gly Ala Glu Tyr Asn
        135                 140                 145

ATA GAG CCA CTT TGG AGA TTT GTT AAT GAT ACC AAA GAC AAA AGA ATG          655
Ile Glu Pro Leu Trp Arg Phe Val Asn Asp Thr Lys Asp Lys Arg Met
    150                 155                 160

TTA GTT TAT AAA TCT GAA GAT ATC AAG AAT GTT TCA CGT TTG CAG TCT          703
Leu Val Tyr Lys Ser Glu Asp Ile Lys Asn Val Ser Arg Leu Gln Ser
165                 170                 175                 180

CCA AAA GTG TGT GGT TAT TTA AAA GTG GAT AAT GAA GAG TTG CTC CCA          751
Pro Lys Val Cys Gly Tyr Leu Lys Val Asp Asn Glu Glu Leu Leu Pro
                185                 190                 195

AAA GGG TTA GTA GAC AGA GAA CCA CCT GAA GAG CTT GTT CAT CGA GTG          799
Lys Gly Leu Val Asp Arg Glu Pro Pro Glu Glu Leu Val His Arg Val
            200                 205                 210

AAA AGA AGA GCT GAC CCA GAT CCC ATG AAG AAC ACG TGT AAA TTA TTG          847
Lys Arg Arg Ala Asp Pro Asp Pro Met Lys Asn Thr Cys Lys Leu Leu
        215                 220                 225
```

FIG. 1(II)

```
GTG GTA GCA GAT CAT CGC TTC TAC AGA TAC ATG GGC AGA GGG GAA GAG          895
Val Val Ala Asp His Arg Phe Tyr Arg Tyr Met Gly Arg Gly Glu Glu
    230                 235                 240

AGT ACA ACT ACA AAT TAC TTA ATA GAG CTA ATT GAC AGA GTT GAT GAC          943
Ser Thr Thr Thr Asn Tyr Leu Ile Glu Leu Ile Asp Arg Val Asp Asp
245                 250                 255                 260

ATC TAT CGG AAC ACT TCA TGG GAT AAT GCA GGT TTT AAA GGC TAT GGA          991
Ile Tyr Arg Asn Thr Ser Trp Asp Asn Ala Gly Phe Lys Gly Tyr Gly
                265                 270                 275

ATA CAG ATA GAG CAG ATT CGC ATT CTC AAG TCT CCA CAA GAG GTA AAA         1039
Ile Gln Ile Glu Gln Ile Arg Ile Leu Lys Ser Pro Gln Glu Val Lys
            280                 285                 290

CCT GGT GAA AAG CAC TAC AAC ATG GCA AAA AGT TAC CCA AAT GAA GAA         1087
Pro Gly Glu Lys His Tyr Asn Met Ala Lys Ser Tyr Pro Asn Glu Glu
        295                 300                 305

AAG GAT GCT TGG GAT GTG AAG ATG TTG CTA GAG CAA TTT AGC TTT GAT         1135
Lys Asp Ala Trp Asp Val Lys Met Leu Leu Glu Gln Phe Ser Phe Asp
    310                 315                 320

ATA GCT GAG GAA GCA TCT AAA GTT TGC TTG GCA CAC CTT TTC ACA TAC         1183
Ile Ala Glu Glu Ala Ser Lys Val Cys Leu Ala His Leu Phe Thr Tyr
325                 330                 335                 340

CAA GAT TTT GAT ATG GGA ACT CTT GGA TTA GCT TAT GTT GGC TCT CCC         1231
Gln Asp Phe Asp Met Gly Thr Leu Gly Leu Ala Tyr Val Gly Ser Pro
                345                 350                 355
```

FIG. 1(III)

```
AGA GCA AAC AGC CAT GGA GGT GTT TGT CCA AAG GCT TAT TAT AGC CCA      1279
Arg Ala Asn Ser His Gly Gly Val Cys Pro Lys Ala Tyr Tyr Ser Pro
            360                 365                 370

GTT GGG AAG AAA AAT ATC TAT TTG AAT AGT GGT TTG ACG AGC ACA AAG      1327
Val Gly Lys Lys Asn Ile Tyr Leu Asn Ser Gly Leu Thr Ser Thr Lys
        375                 380                 385

AAT TAT GGT AAA ACC ATC CTT ACA AAG GAA GCT GAC CTG GTT ACA ACT      1375
Asn Tyr Gly Lys Thr Ile Leu Thr Lys Glu Ala Asp Leu Val Thr Thr
    390                 395                 400

CAT GAA TTG GGA CAT AAT TTT GGA GCA GAA CAT GAT CCG GAT GGT CTA      1423
His Glu Leu Gly His Asn Phe Gly Ala Glu His Asp Pro Asp Gly Leu
405                 410                 415                 420

GCA GAA TGT GCC CCG AAT GAG GAC CAG GGA GGG AAA TAT GTC ATG TAT      1471
Ala Glu Cys Ala Pro Asn Glu Asp Gln Gly Gly Lys Tyr Val Met Tyr
                425                 430                 435

CCC ATA GCT GTG AGT GGC GAT CAC GAG AAC AAT AAG ATG TTT TCA AAC      1519
Pro Ile Ala Val Ser Gly Asp His Glu Asn Asn Lys Met Phe Ser Asn
            440                 445                 450

TGC AGT AAA CAA TCA ATC TAT AAG ACC ATT GAA AGT AAG GCC CAG GAG      1567
Cys Ser Lys Gln Ser Ile Tyr Lys Thr Ile Glu Ser Lys Ala Gln Glu
        455                 460                 465

TGT TTT CAA GAA CGC AGC AAT AAA GTT TGT GGG AAC TCG AGG GTG GAT      1615
Cys Phe Gln Glu Arg Ser Asn Lys Val Cys Gly Asn Ser Arg Val Asp
    470                 475                 480
```

FIG. 1(IV)

```
GAA GGA GAA GAG TGT GAT CCT GGC ATC ATG TAT CTG AAC AAC GAC ACC      1663
Glu Gly Glu Glu Cys Asp Pro Gly Ile Met Tyr Leu Asn Asn Asp Thr
485                 490                 495                 500

TGC TGC AAC AGC GAC TGC ACG TTG AAG GAA GGT GTC CAG TGC AGT GAC      1711
Cys Cys Asn Ser Asp Cys Thr Leu Lys Glu Gly Val Gln Cys Ser Asp
                505                 510                 515

AGG AAC AGT CCT TGC TGT AAA AAC TGT CAG TTT GAG ACT GCC CAG AAG      1759
Arg Asn Ser Pro Cys Cys Lys Asn Cys Gln Phe Glu Thr Ala Gln Lys
            520                 525                 530

AAG TGC CAG GAG GCG ATT AAT GCT ACT TGC AAA GGC GTG TCC TAC TGC      1807
Lys Cys Gln Glu Ala Ile Asn Ala Thr Cys Lys Gly Val Ser Tyr Cys
        535                 540                 545

ACA GGT AAT AGC AGT GAG TGC CCG CCT CCA GGA AAT GCT GAA GAT GAC      1855
Thr Gly Asn Ser Ser Glu Cys Pro Pro Pro Gly Asn Ala Glu Asp Asp
    550                 555                 560

ACT GTT TGC TTG GAT CTT GGC AAG TGT AAG GAT GGG AAA TGC ATC CCT      1903
Thr Val Cys Leu Asp Leu Gly Lys Cys Lys Asp Gly Lys Cys Ile Pro
565                 570                 575                 580

TTC TGC GAG AGG GAA CAG CAG CTG GAG TCC TGT GCA TGT AAT GAA ACT      1951
Phe Cys Glu Arg Glu Gln Gln Leu Glu Ser Cys Ala Cys Asn Glu Thr
                585                 590                 595

GAC AAC TCC TGC AAG GTG TGC TGC AGG GAC CTT TCT GGC CGC TGT GTG      1999
Asp Asn Ser Cys Lys Val Cys Cys Arg Asp Leu Ser Gly Arg Cys Val
            600                 605                 610
```

FIG. 1(V)

```
CCC TAT GTC GAT GCT GAA CAA AAG AAC TTA TTT TTG AGG AAA GGA AAG      2047
Pro Tyr Val Asp Ala Glu Gln Lys Asn Leu Phe Leu Arg Lys Gly Lys
        615                 620                 625

CCC TGT ACA GTA GGA TTT TGT GAC ATG AAT GGC AAA TGT GAG AAA CGA      2095
Pro Cys Thr Val Gly Phe Cys Asp Met Asn Gly Lys Cys Glu Lys Arg
    630                 635                 640

GTA CAG GAT GTA ATT GAA CGA TTT TGG GAT TTC ATT GAC CAG CTG AGC      2143
Val Gln Asp Val Ile Glu Arg Phe Trp Asp Phe Ile Asp Gln Leu Ser
645                 650                 655                 660

ATC AAT ACT TTT GGA AAG TTT TTA GCA GAC AAC ATC GTT GGG TCT GTC      2191
Ile Asn Thr Phe Gly Lys Phe Leu Ala Asp Asn Ile Val Gly Ser Val
                665                 670                 675

CTG GTT TTC TCC TTG ATA TTT TGG ATT CCT TTC AGC ATT CTT GTC CAT      2239
Leu Val Phe Ser Leu Ile Phe Trp Ile Pro Phe Ser Ile Leu Val His
            680                 685                 690

TGT GTG GAT AAG AAA TTG GAT AAA CAG TAT GAA TCT CTG TCT CTG TTT      2287
Cys Val Asp Lys Lys Leu Asp Lys Gln Tyr Glu Ser Leu Ser Leu Phe
        695                 700                 705

CAC CCC AGT AAC GTC GAA ATG CTG AGC AGC ATG GAT TCT GCA TCG GTT      2335
His Pro Ser Asn Val Glu Met Leu Ser Ser Met Asp Ser Ala Ser Val
    710                 715                 720

CGC ATT ATC AAA CCC TTT CCT GCG CCC CAG ACT CCA GGC CGC CTG CAG      2383
Arg Ile Ile Lys Pro Phe Pro Ala Pro Gln Thr Pro Gly Arg Leu Gln
725                 730                 735                 740
```

FIG. 1(VI)

```
CCT GCC CCT GTG ATC CCT TCG GCG CCA GCA GCT CCA AAA CTG GAC CAC      2431
Pro Ala Pro Val Ile Pro Ser Ala Pro Ala Ala Pro Lys Leu Asp His
                745                 750                 755

CAG AGA ATG GAC ACC ATC CAG GAA GAC CCC AGC ACA GAC TCA CAT ATG      2479
Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Met
            760                 765                 770

GAC GAG GAT GGG TTT GAG AAG GAC CCC TTC CCA AAT AGC AGC ACA GCT      2527
Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Thr Ala
        775                 780                 785

GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACC AGA AGT GAA      2575
Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu
    790                 795                 800

AAG GCT GCC TCC TTT AAA CTG CAG CGT CAG AAT CGT GTT GAC AGC AAA      2623
Lys Ala Ala Ser Phe Lys Leu Gln Arg Gln Asn Arg Val Asp Ser Lys
805                 810                 815                 820

GAA ACA GAG TGC TAA TTTAGTTCTC AGCTCTTCTG ACTTAAGTGT GCAAAATATT      2678
Glu Thr Glu Cys ***

TTTATAGATT TGACCTACAA TCAATCACAG CTTATATTTT GTGAAGACTG GGAAGTGACT     2738

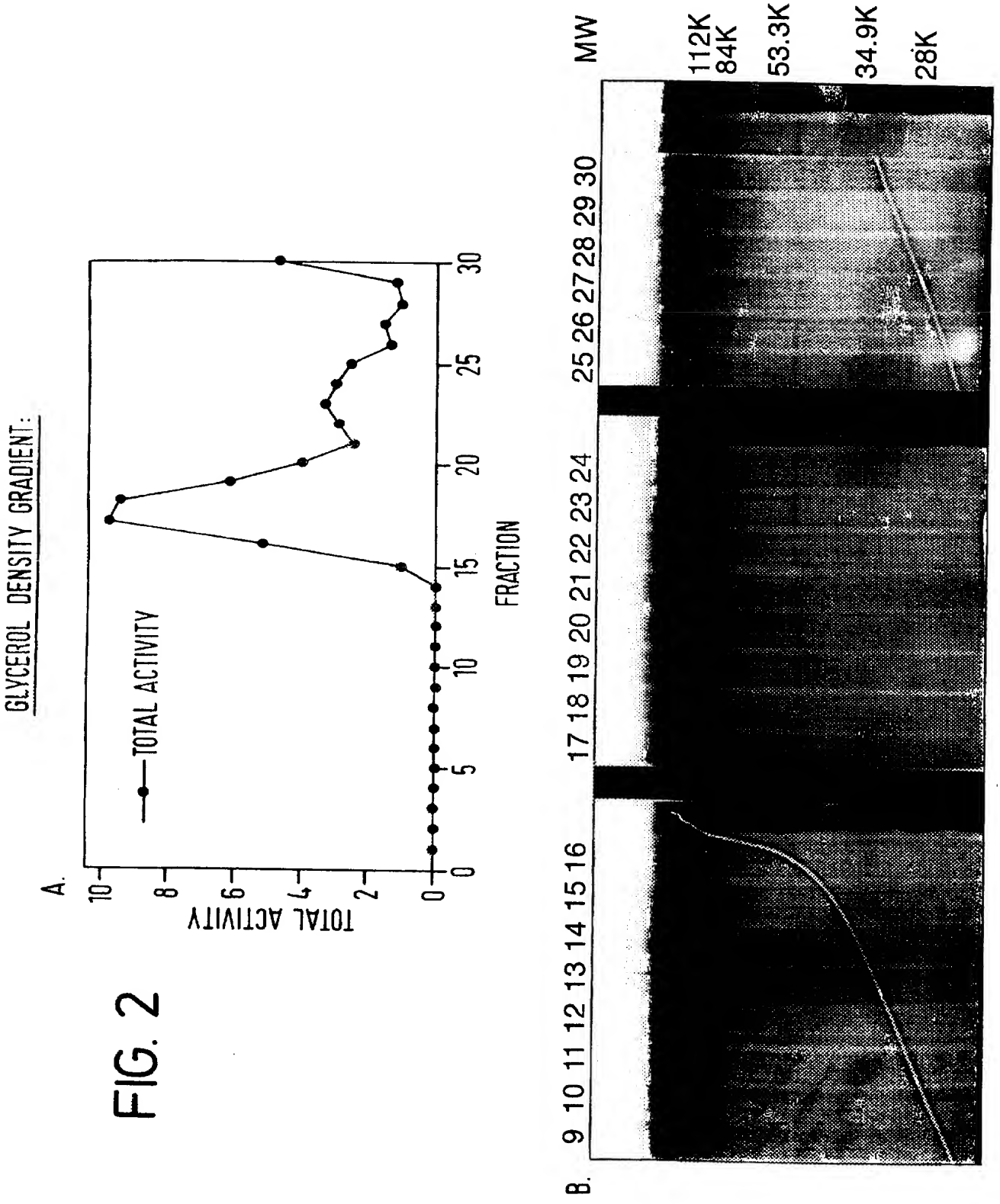
TAGCAGATGC TGGTCATGTG TTTGGAACTT CCTGCAGGTA AACAGTTCTT GTGTGGGTTT     2798

GGGCCCNTCT CCTTTTGGAA AAGGTAAGGG TGAAGGTGAA TCTTGCTTAT TNTGGGGGTT     2858

TCAGGTTTNA GTTTTTAAAA TATCTTTTGG ACCTGTGGGT GNAAAAGCAG AAATACAGNT     2918

GGATTGGGTT ATGAGTATTT ACGT                                            2942
```

FIG. 1(VII)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TOTAL ACTIVITY
TOTAL ACTIVITY
FRACTION
0
2
4
6
8
10
0
5
10
15
20
25
30
B.
MW
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84K
53.3K
34.9K
28K
9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

FIG. 2

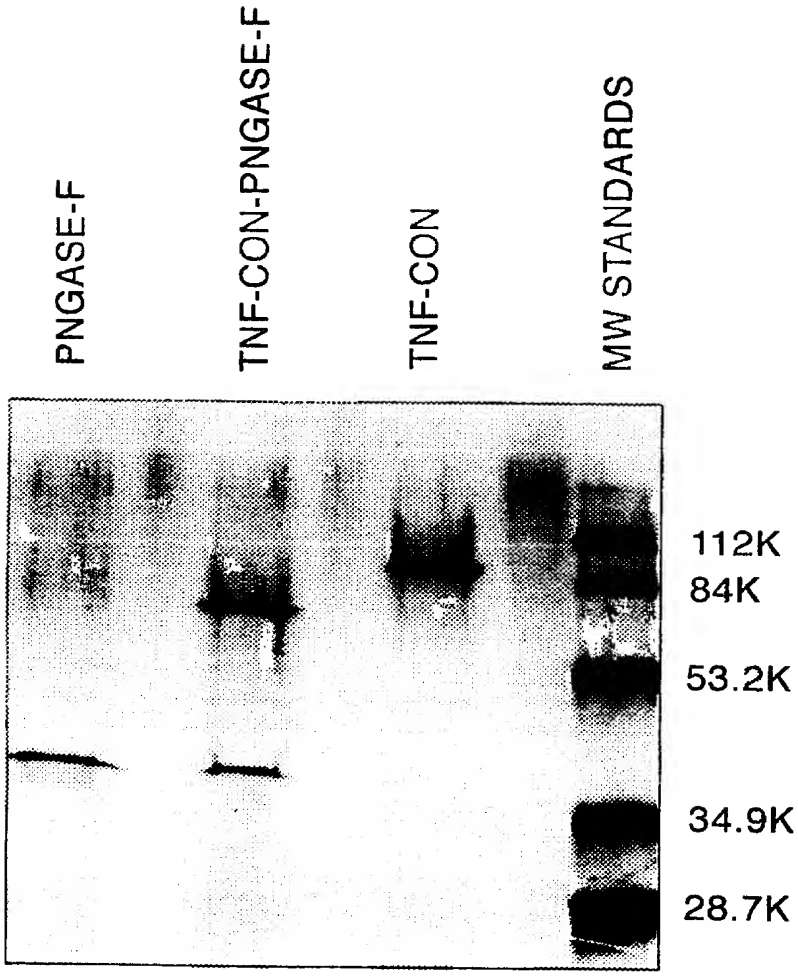
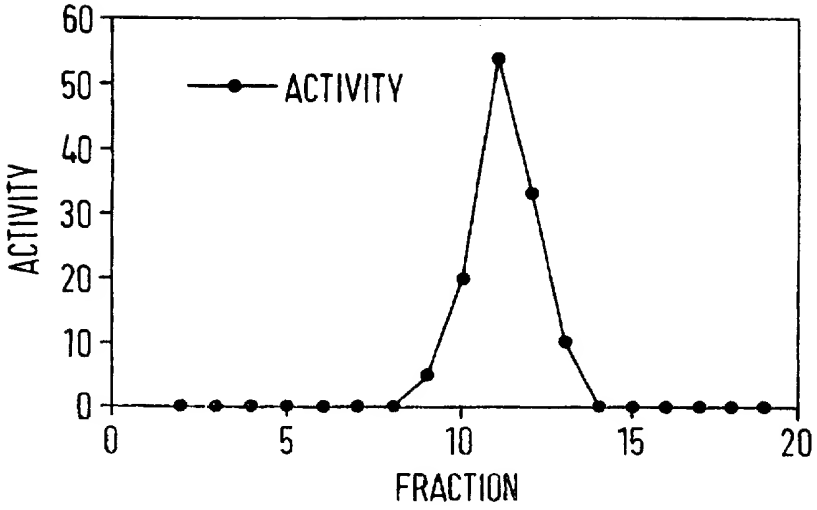

PNGASE-F
TNF-CON-PNGASE-F
TNF-CON
MW STANDARDS
112K
84K
53.2K
34.9K
28.7K

FIG. 3

A.



B.

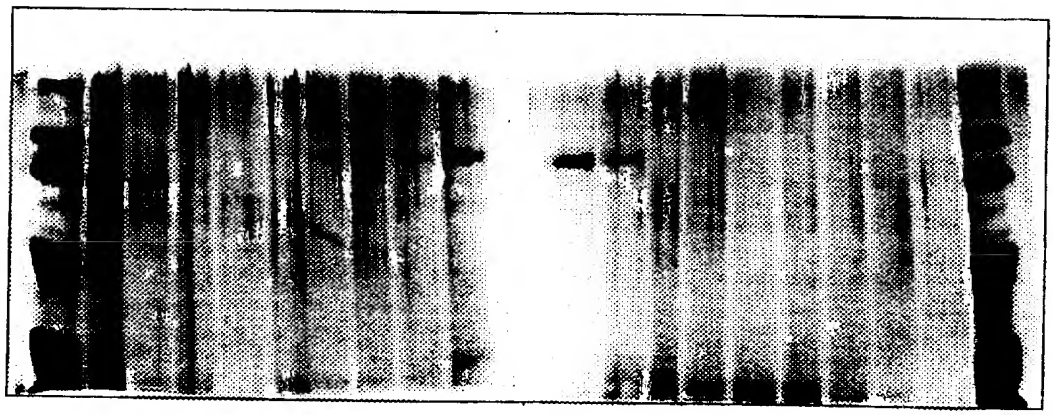
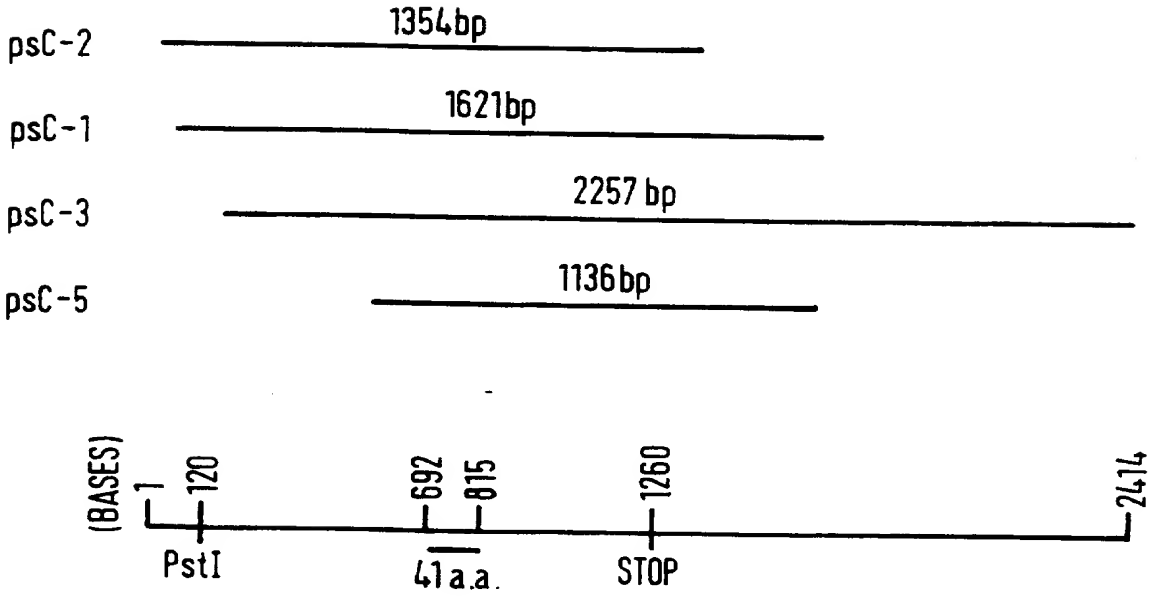


FIG. 4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2257 bp
psC-5
1136bp
(BASES)
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815
1260
2414
PstI
41 a.a.
STOP

FIG. 5

```
GAA GCT GAC CTT GTG ACA ACT CAT GAA TTG GGG CAC AAT TTT GGA GCA      48
Glu Ala Asp Leu Val Thr Thr His Glu Leu Gly His Asn Phe Gly Ala

GAA CAC GAT CCA GAT GGT TTA GCA GAA TGT GCC CCA AAC GAG GAC CAG      96
Glu His Asp Pro Asp Gly Leu Ala Glu Cys Ala Pro Asn Glu Asp Gln

GGA GGA AAA TAC GTC ATG TAT CCC ATA GCC GTG AGT GGT GAT CAT GAG     144
Gly Gly Lys Tyr Val Met Tyr Pro Ile Ala Val Ser Gly Asp His Glu

AAC AAC AAG ATG TTT TCA AAC TGC AGT AAA CAG TCC ATC TAT AAG ACC     192
Asn Asn Lys Met Phe Ser Asn Cys Ser Lys Gln Ser Ile Tyr Lys Thr

ATT GAA AGT AAG GCC CAG GAG TGT TTT CAA GAG CGC AGC AAC AAA GTG     240
Ile Glu Ser Lys Ala Gln Glu Cys Phe Gln Glu Arg Ser Asn Lys Val

TGT GGC AAC TCC AGG GTG GAT GAG GGG GAG GAG TGC GAC CCC GGC ATC     288
Cys Gly Asn Ser Arg Val Asp Glu Gly Glu Glu Cys Asp Pro Gly Ile

ATG TAC CTG AAC AAC GAC ACC TGC TGC AAC AGC GAC TGC ACC CTG AGG     336
Met Tyr Leu Asn Asn Asp Thr Cys Cys Asn Ser Asp Cys Thr Leu Arg

CCG GGC GTC CAG TGC AGT GAT AGG AAC AGT CCT TGC TGT AAA AAC TGT     384
Pro Gly Val Gln Cys Ser Asp Arg Asn Ser Pro Cys Cys Lys Asn Cys

CAG TTC GAG ACG GCC CAG AAG AAG TGC CAG GAG GCT ATT AAT GCC ACT     432
Gln Phe Glu Thr Ala Gln Lys Lys Cys Gln Glu Ala Ile Asn Ala Thr

TGC AAA GGC GTG TCT TAC TGC ACA GGT AAC AGC AGT GAG TGC CCC CCT     480
Cys Lys Gly Val Ser Tyr Cys Thr Gly Asn Ser Ser Glu Cys Pro Pro

CCG GGA AAC GCC GAG GAC GAC ACG GTG TGC CTG GAC CTG GGC AGG TGC     528
Pro Gly Asn Ala Glu Asp Asp Thr Val Cys Leu Asp Leu Gly Arg Cys
```

FIG. 6(I)

```
AAG GAC GGC AAG TGC GTG CCC TTC TGC GAG CGG GAG CAG CGG CTG GAG      576
Lys Asp Gly Lys Cys Val Pro Phe Cys Glu Arg Glu Gln Arg Leu Glu

TCC TGC GCG TGT AAC GAA ACC GAC CAC TCG TGC AAG GTG TGC TGC CGG      624
Ser Cys Ala Cys Asn Glu Thr Asp His Ser Cys Lys Val Cys Cys Arg

GCC CCC TCG GGC CGT TGG CTG CCC TAC GTG GAC GCC GAA CAG AAG AAC      672
Ala Pro Ser Gly Arg Trp Leu Pro Tyr Val Asp Ala Glu Gln Lys Asn

TTG TTT TTG AGG AAG GGG AAG CCC TGT ACA GTA GGA TTT TGT GAC ATG      720
Leu Phe Leu Arg Lys Gly Lys Pro Cys Thr Val Gly Phe Cys Asp Met

AAT GGC AAG TGT GAG AAG CGA GTG CAG GAC GTC ATC GAG CGG TTC TGG      768
Asn Gly Lys Cys Glu Lys Arg Val Gln Asp Val Ile Glu Arg Phe Trp

GAG TTC ATT GAC AAG CTG AGC ATC AAT ACT TTC GGG AAG TTC CTG GCA      816
Glu Phe Ile Asp Lys Leu Ser Ile Asn Thr Phe Gly Lys Phe Leu Ala

GAC AAC ATC GTG GGC TCC GTC CTG GTG TTC TCC CTG ATG CTC TGG ATC      864
Asp Asn Ile Val Gly Ser Val Leu Val Phe Ser Leu Met Leu Trp Ile

CCC GTC AGC ATC CTC GTC CAC TGC GTG GAT AAG AAG CTG GAT AAG CAG      912
Pro Val Ser Ile Leu Val His Cys Val Asp Lys Lys Leu Asp Lys Gln

TAC GAA TCC CTG TCT CTG CTG CAC CCC AGC AAC GTG GAG ATG CTA AGC      960
Tyr Glu Ser Leu Ser Leu Leu His Pro Ser Asn Val Glu Met Leu Ser

AGC ATG GAT TCA GCA TCC GTT CGC ATC ATC AAG CCC TTT CCT GCG CCC     1008
Ser Met Asp Ser Ala Ser Val Arg Ile Ile Lys Pro Phe Pro Ala Pro
```

FIG. 6(II)

```
CAG ACC CCA GGC CGC CTG CAG CCC CTG CAG CCC CTG CAG CCC GGC CCC      1056
Gln Thr Pro Gly Arg Leu Gln Pro Leu Gln Pro Leu Gln Pro Gly Pro

GTG CTG CCC TCT GCG CCT TCG GTG CCC GTG GCT CCA AAA CTG GAC CAC      1104
Val Leu Pro Ser Ala Pro Ser Val Pro Val Ala Pro Lys Leu Asp His

CAG CGG ATG GAC ACC ATC CAG GAG GAC CCC AGC ACG GAC TCG CAC GTG      1152
Gln Arg Met Asp Thr Ile Gln Glu Asp Pro Ser Thr Asp Ser His Val

GAC GAG GAC GGC TTC GAG AAG GAC CCT TTC CCC AAC AGC AGT GCC GCT      1200
Asp Glu Asp Gly Phe Glu Lys Asp Pro Phe Pro Asn Ser Ser Ala Ala

GCC AAG TCA TTT GAG GAT CTC ACG GAC CAT CCG GTC ACG AGA AGT GAA      1248
Ala Lys Ser Phe Glu Asp Leu Thr Asp His Pro Val Thr Arg Ser Glu

AAG GCC TCG TCC TTT AAG CTG CAG CGC CAG AGT CGC GTT GAC AGC AAG      1296
Lys Ala Ser Ser Phe Lys Leu Gln Arg Gln Ser Arg Val Asp Ser Lys

GAA ACG GAG TGC TAGTTCAGGG CTGCGTCCGG AGCTCTCGGA CTTGGCGTGC         1348
Glu Thr Glu Cys ***

AGGATATTTT TATAGATTTG ACCTCAGATC ACTGCGGAGA TCTTGTGAAG ATTTGGGAGA   1408

AAAAAGGAAG TGACTTCCCA GCAGATGCTG GTCATGAGTT TGGACTTCCT CCACCTCCGT   1468

GAGACTGTGT GGGTAGCCCT TTTCCTTTGA AGAGGTAAGG CGAACCTAGC TTACTTTGAG   1528

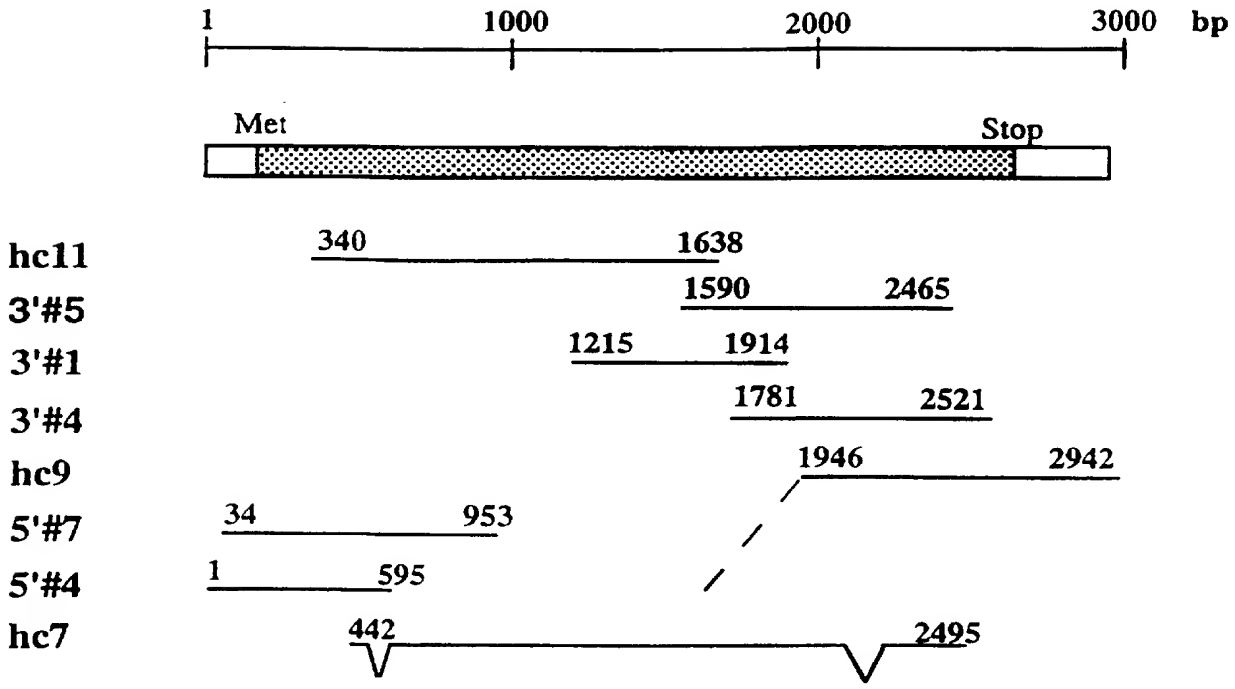
GCCTTCAGGT TTTAGTCTTT TGATCTTTAA AATATCTTTC AACCTGTGGT GCAGAAGCAG   1588

AAACCACAGC TGGATTATGT TATGACTATT TACGTTTTTG TAAATTACCT TTATATTGAG   1648
```

FIG. 6(III)

```
AACAGCACTG ATTGTGGAGG TGATCATGTT TTTAAGACAC TGTAACGATC CAATGAACAC    1708
AAAGAGGCAT TTCATCAGTT TCCTGCGAGG ATAACTGGAA CACAGAATAG ATGGATTTCT    1768
TCCCTTCAGG TCAAATAAAG TCCGAGGTGC GTAATGTCAC CCAAATCTGC TTTCTGATGC    1828
AGAACCCGGT TCTACGTTCC CCCCAAAGGT GGGTTCAGGT TCGAGGTATT GCCTAGAACT    1888
TAGAGCAGAA GGGAGACCAC ACATCTGAAT TTAGGAATAA ACTGGGTTAA TGTTTCTTCC    1948
TGTTTCTAAA TGTTCAGAAT TTCTAATTTC TAGAATTAAA ACAATCTAGC TTTATAGTAA    2008
AGATTATAAA TTATAGCCAC ATGAATTGAC AGAATCCTGC TGATTAACTG TTTAGGGCCA    2068
TACCTAGCTG GGCAGTTTTT CTGGGTTTTG TAAGTATCTT CCTGTCTCTC TACGGGTGCC    2128
ACGCTCCTAA AAAACCCACC ATCCACAAAA CCTCAACCCT TCAGAACAGG AGGACGGTCG    2188
CCTCTTCGCA AACACTAGTG GCCCAGACGG TGCGTGCGGT TGGGCGCGCT TGCCAGGCGA    2248
ACAGGGTGCG AGGCGCTGGC CGGGGGTCTG TCCTCAGACC ATTGGTCCCT AGACCCTAGA    2308
CCTTCTGGCT CGCGTGCTTC GGCACCGTCT GCGCGCCCTG CGCGAGGGAT AAAAACCATC    2368
CGGCTGAGTT CTGGTTTCAA AAGCTTTTTC CCCTGCTAAG GACTGCAGTG TGCTCACCAG    2428
CCAGCACCCC TTAACAGGTA ACTTGTAAGC TTCGAG                              2464
```

FIG. 6(IV)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2000
3000
bp
Met
Stop
hc11
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1638
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3'#1
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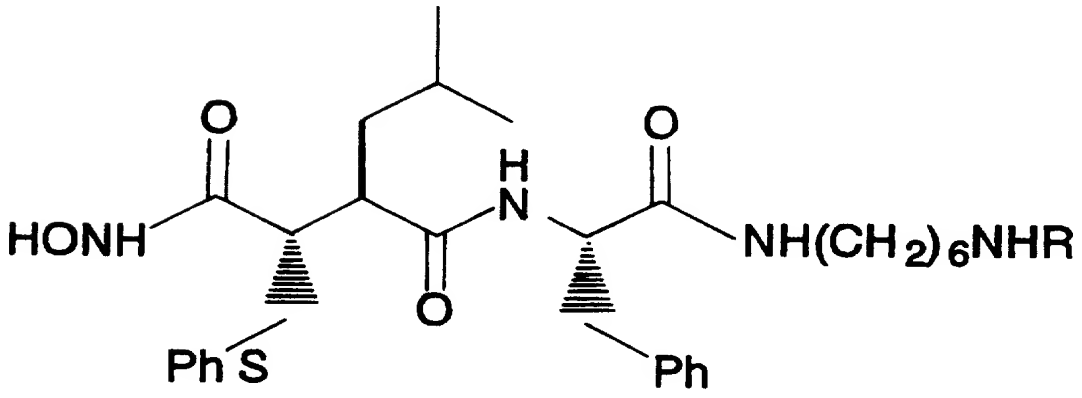
1914
3'#4
1781
2521
hc9
1946
2942
5'#7
34
953
5'#4
1
595
hc7
442
2495

FIG. 7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HONH
H
N
O
O
NH(CH2)6NHR
PhS
Ph

FIG. 8

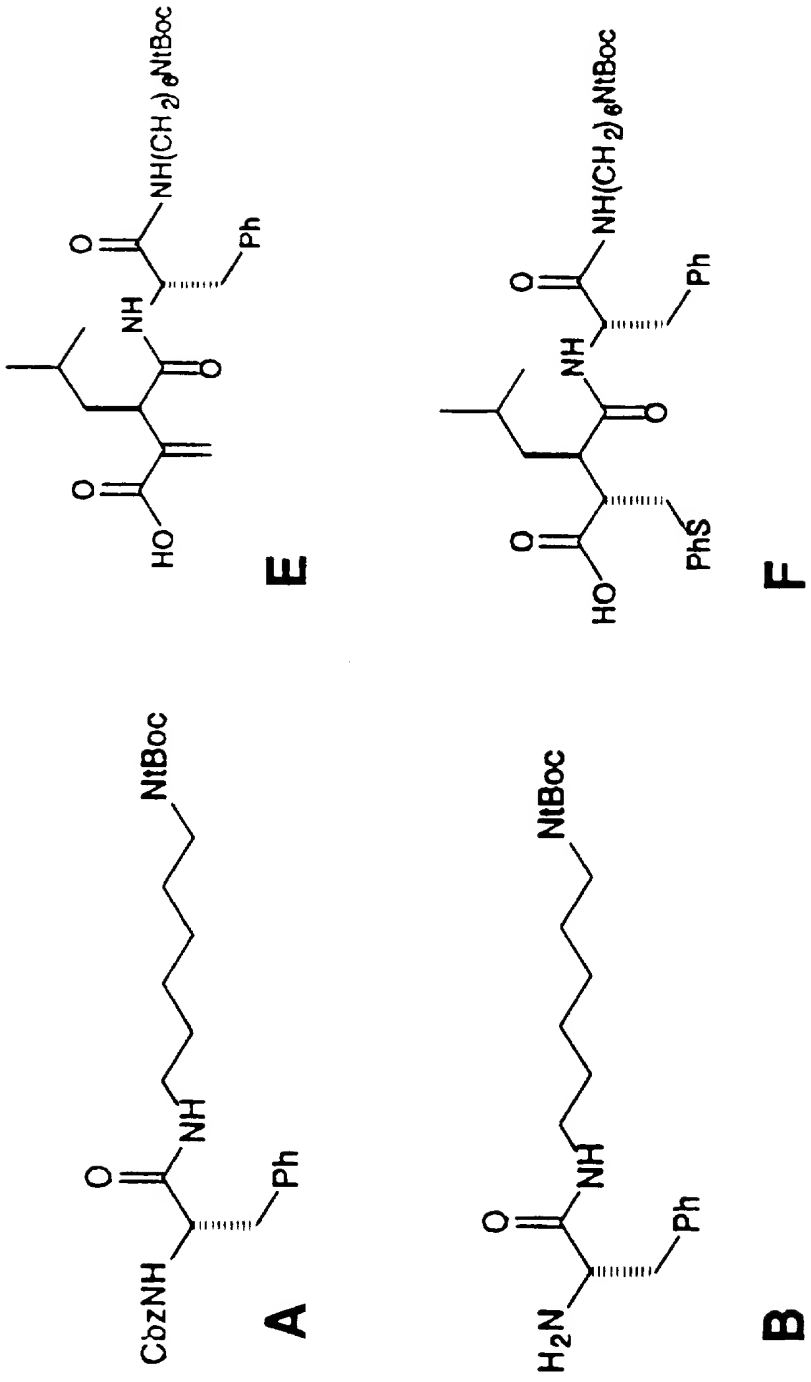

NtBoc
NH
Ph
CbzNH
O
A
NtBoc
NH
Ph
H2N
O
B
NH(CH2)6NtBoc
NH
Ph
HO
O
E
NH(CH2)6NtBoc
NH
Ph
PhS
HO
O
F

FIG. 9(I)

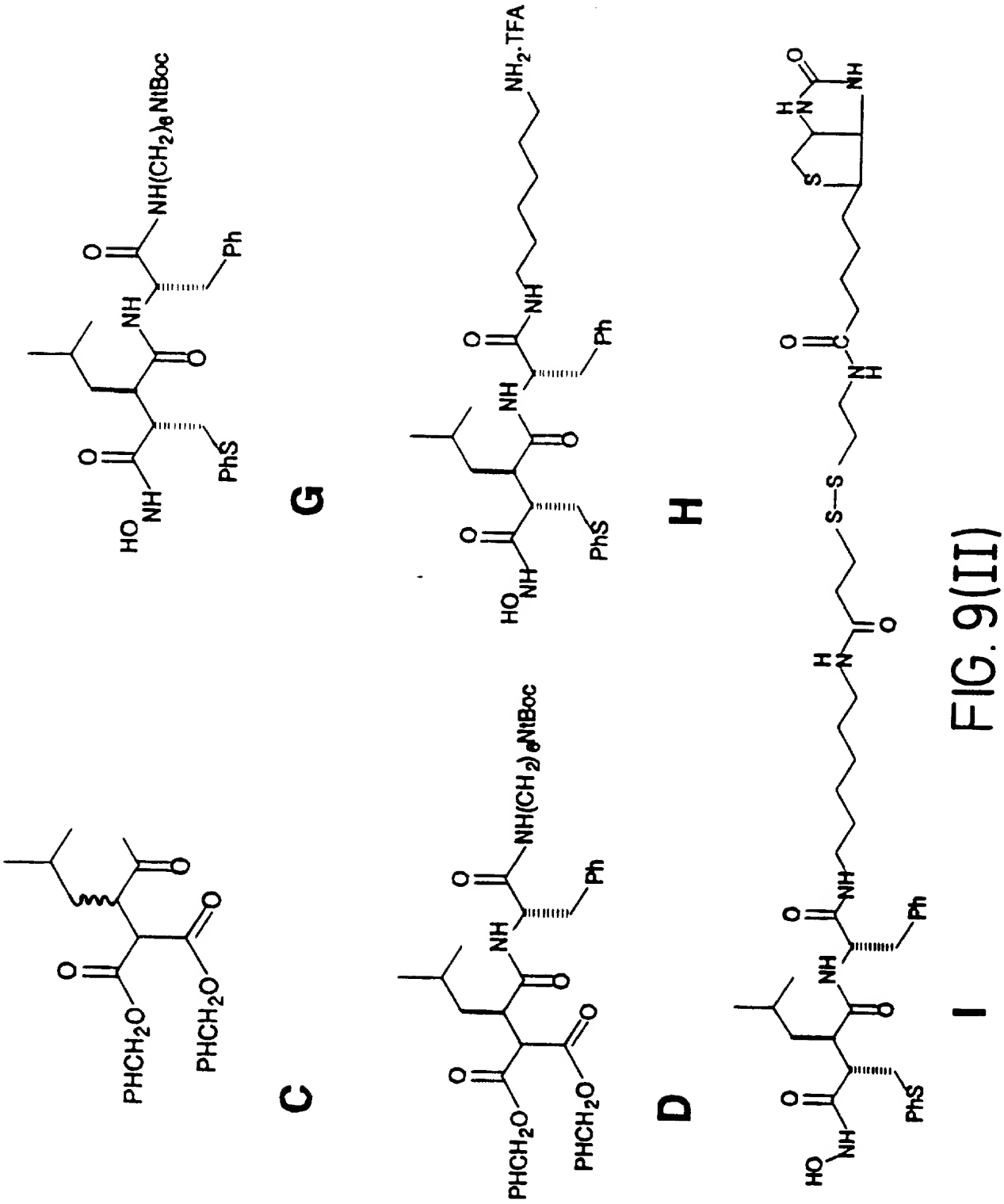
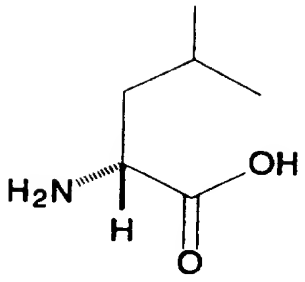
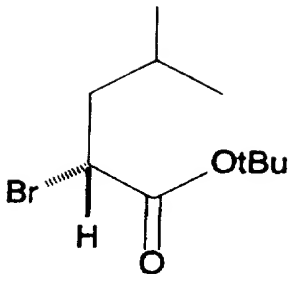


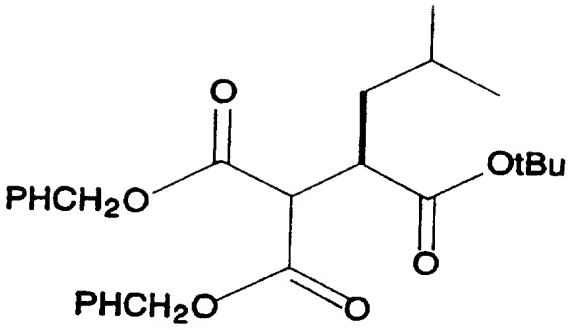
FIG. 9(II)


H2N
H
OH
O

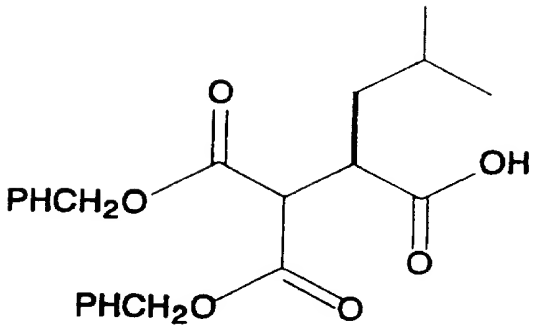
A


Br
H
OtBu
O

B


PHCH2O
PHCH2O
O
O
O
OtBu

C


PHCH2O
PHCH2O
O
O
O
OH

D

FIG. 10

```
AAACTGGCTG AACCGGCCTT TTGGTAACGC CACCTTCACT TCTGGGGGCG TCGAACCTGG    60

CGGTAGAATC TTCCCAGTAG GCGGCGCGGG AGGGAAAAGA GGATTGAGGG GCTAGGCCGG   120

GCGGATCCCG TCCTCCCCCG ATGTGAGCAG TTTTCCGAAA CCCCGTCAGG CGAAGGCTGC   180

CCAGAGAGGT GGAGTCGGTA GCGGGGCCGG GAACATGAGG CAGTCTCTCC TATTCCTGAC   240

CAGCGTGGTT CCTTTCGTGC TGGCGCCGCG ACCTCCGGAT GACCCGGGCT TCGGCCCCCA   300

CCAGAGACTC GAGGAGCTTG ATTCTTTGCT CTCAGACTAC GATATTCTCT CTTTATCTAA   360

TATCCAGCAG CATTCGGTAA GAAAAAGAGA TCTACAGACT TCAACACATG TAGAAACACT   420

ACTAACTTTT TCAGCTTTGA AAAGGCATTT TAAATTATAC CTGACATCAA GTACTGAACG   480

TTTTTCACAA AATTTCAAGG TCGTGGTGGT GGATGGTA                           518
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FIG. 11